University of Alberta

Antigenic Variation in *Bordetella pertussis*

by

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©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY
EDMONTON, ALBERTA

SPRING 2001

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DEDICATION

This thesis is dedicated to some very special people in my life. First to my parents and siblings, for their constant love, support, and understanding that helped me through all the years. And to Dino, the love of my life, thank-you for the encouragement, and the love, no matter how difficult things got. Knowing how proud you are of me, no matter what, is the fuel that keeps me going. Without you, I don't think I would have made it. With you, I think I could do anything.

ABSTRACT

This thesis describes the use of single-strand conformation polymorphism (SSCP) as a technique to screen *Bordetella pertussis* strains isolated in Alberta and Québec between 1985 and 1994 for antigenic variants of pertactin and the S1 subunit of pertussis toxin. Compared to strains from The Netherlands, the United States, Finland and Italy, our Canadian isolates were found to be in a state of change from "old" to "new" types, with strains containing two "old" antigens, strains with one "old" antigen and one "new" antigen, and strains with two "new" antigens. Clinical strains were found to be antigenically different, in their *prn* and *ptxS1* types, from the Connaught whole cell vaccine strains. A new pertactin type $prn9_1$ was found in Canadian isolates. Two of the Connaught strains were also found to have a single base sequence variation in adenylate cyclase that resulted in an amino acid change at residue 892.

Antigenic typing, as presented in this thesis, can be used as a foundation for future epidemiological studies of *Bordetella pertussis* in Canada. It may also help to explain why pertussis is not being controlled, despite adequate immunization.

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ABBREVIATIONS

AC	Adenylate cyclase
ACV	Acellular vaccine
A-F	Armand-Frappier
AGG 2	Agglutinogen 2
AGG 3	Agglutinogen 3
CE	Capillary electrophoresis
CMI	Cell-mediated immunity
CyaA	Adenylate cyclase toxin
DTPa	Diphtheria-tetanus-pertussis (acellular)
DT	Diphtheria-tetanus
DTPw	Diphtheria-tetanus-pertussis (whole-cell)
FHA	Filamentous haemagglutinin
FIM	Fimbriae
Fim 2	Fimbrial major subunit 2
Fim 3	Fimbrial major subunit3
Fim D	Fimbrial minor subunit D
MAbs	Monoclonal antibodies
OMP	Outer membrane protein
OMP40	Major porin
Omp40	Major porin gene
PCR	Polymerase chain reaction
PRN	Pertactin
prn	Pertactin gene
PT	Pertussis toxin
ptxS1	pertussis toxin S1 subunit
RGD	Arginine-Glycine-Aspartic acid motif
SSCP	Single-strand conformation polymorphism
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Th1	T-helper response 1 (cell-mediated)
Th2	T-helper response 2 (humoral)
WCV	Whole-cell vaccine
WHO	World Health Organization

CHAPTER I

GENERAL INTRODUCTION

Bordetella pertussis, the etiological agent of whooping cough, is a small, gram-negative coccobacillus. *B. pertussis* is a strict aerobe that requires a humid environment and a special media enriched with blood in order to grow. In 1906, Bordet and Gengou of the Pasteur Institute of Brussels first isolated the causative agent of whooping cough and called it *Haemophilus pertussis* because of its fastidious growth needs and similar appearance to the *Haemophilus sp.* (Bordet, J., 1906). Subsequently, in 1940, Hornibrook showed that the X and V factors in blood necessary for the growth of the genus *Haemophilus* were not required for the growth of *H. pertussis* (Hornibrook, J. W., 1940). This led to the creation of a new genus *Bordetella*, in honor of Bordet.

Clinical Manifestations of Whooping Cough

B. pertussis is a strict human pathogen. Guillaume de Baillou made the first recorded description of whooping cough in Paris in 1578 (Howson, C. P., 1991). The most severe form of the disease occurs in children under the age of 1 year and is characterized by three phases (Kerr, J. R., 2000, Mahon, C. R., 1996, Walker, E., 1988). Following exposure, usually by aerosol droplets from an infected person, there is a 5-7 day incubation period that is asymptomatic. The first stage, lasting about 7-14 days, is

the catarrhal phase consisting of non-specific cold-like symptoms including rhinitis, a low- grade fever, and a non-productive cough. Recovery rate of the organism at this stage is very high, up to 90%. The catarrhal stage is followed by the second paroxysmal stage, which lasts approximately 2 weeks. During this stage, the now productive cough becomes more severe and paroxysmal. Several times a day, the child may be plagued with a series of several short coughs followed by an inspiration of air against a partially closed glottis producing the characteristic "whoop". The child may experience cyanosis during the paroxysm, which may be followed by vomiting. Other consequences of the paroxysmal coughing may be rupturing of the frenulum of the tongue, prolonged increased pressure in the abdominal and thoracic cavities with subconjunctival hemorrhage, epistaxis, petechiae, brain hemorrhage, spontaneous pneumothorax, inguinal hernia, and rectal prolapse (Kerr, J. R., 2000, Walker, E., 1988). The recovery rate of the organism during the paroxysmal stage is much reduced, therefore it is thought that most of the clinical symptoms of this stage may be caused by the release and dissemination of the many toxins produced by *B. pertussis* (Kerr, J. R., 2000). In the final convalescent stage lasting 2 weeks to several months, the paroxysms begin to lessen and the severity of the coughs taper, although a nonparoxysmal cough may persist.

Although this is the characteristic clinical description, it is rarely the most common presentation. Even in severe infections, the paroxysms may be infrequent or may not occur at all. Often in adults and adolescents, or in cases of vaccine failure, the presentation is subclinical and goes undiagnosed (Long, S., 1990).

Whole-Cell Vaccines

Almost as soon as Bordet and Gengou published their data on the causative agent of whooping cough in 1906, experiments with killed whole-cell vaccines began (WCV). WCV were developed and used in children by Bordet and Gengou in 1912 in Brussels, Charles Nicolle of the Pasteur Institute, in Tunis in 1913, and Thorwald Madsen of the Danish State Serum Institute in Copenhagen in 1914, who used his WCV for the first time on a large scale in 1925 (Howson, C. P., 1991). In 1957 mass inoculation of children began in the United Kingdom, although immunization began on a local scale in 1942 (Howson, C. P., 1991). Immunization in Canada and the United States began in 1943 and 1944 respectively (Howson, C. P., 1991). In 1947, the American Academy of Pediatrics suggested the routine use of the whole-cell vaccine in the form of the diphtheria-tetanus-pertussis combination (DTPw) (Howson, C. P., 1991). In the mid-1960s many States in the U.S. passed laws making it mandatory that all children must have DTPw before they enter school (Howson, C. P., 1991).

Most of the first WCVs were crude concoctions, but a number of discoveries lead to the creation of new and improved vaccines. The first was made in 1931 by Leslie and Gardner, when they noted that *B. pertussis* underwent a genotypic phase variation during subculture (Preston, N. W., 1988, Stainer, D. W., 1988). Fresh cultures were "smooth" and virulent, which they called phase I and II. After prolonged subculture, the colonies became "rough" and lost virulence and antigenic factors, which they called phases III and IV. Therefore, current vaccines are only made from phase 1 or "fresh" strains (Stainer, D. W., 1988).

In 1967, Cameron discovered that strains and colonies that appeared to be homogenous were not, but showed differing agglutination patterns and hemolytic activity, which allowed him to group them into four patterns that were serotype specific and generated antibodies that agglutinated the bacteria (Cameron, J., 1967, Preston, N. W., 1988, Stainer, D. W., 1988). This difference in agglutination patterns happened through a spontaneous change that occurred with either a loss or gain of agglutinogen 2 (AGG 2) or agglutinogen 3 (AGG 3), resulting in the parent serotype of 1,2,3, (meaning that the parent strain contained all three agglutinogens) changing to type 1,2 or type 1,3 or the degraded type of 1, during serial subcultures of single colonies. Since mass vaccination in the United Kingdom began before Cameron's discovery, most vaccines were made from fresh isolates of the 1,2 serotype. Serotypes 1,2 and 1,2,3 were predominant before 1958 and by 1963 serotype 1,3 prevailed (Preston, N. W., 1988). This lead to decreased vaccine efficacy during an epidemic in 1963-64. Subsequently, all serotypes were included in vaccines used in the United Kingdom after 1966, and in 1979 the WHO recommended that strains used for the vaccines should contain all of the serotype antigens (Preston, N. W., 1988).

Since the implementation of the WCV, there has been a substantial decline in mortality and morbidity due to pertussis (Fine, P. E., 1988, Howson, C. P., 1991). Despite this, vaccine efficacy has been controversial throughout the history of the WCV. Whole-cell vaccine efficacy can be demonstrated through household contact studies. Depending on the study, the vaccine preparation and country, and case definition, vaccine efficacy has been shown to be between 59 to 97% (Cherry, J., 1996, 1992, Guris, D., 1997, Onorato, I., 1992, Palmer, S., 1991), but overall, it is believed the WCV is more

than 80% efficacious (Kerr, J. R., 2000). Despite this, there have been incidences of WCVs with substandard activity and efficacy, such as described Nova Scotia and Sweden (Halperin, S., 1989, Howson, C. P., 1991). This, in turn, prompted termination of the vaccination program in Sweden (Halperin, S., 1989, Howson, C. P., 1991).

Vaccine safety and reactogenicity has also been an issue through out the history of the WCV. Reactogenicity is the potential of the vaccine to cause adverse reactions in the recipients (see below, page 17). In 1974, a widely publicized fatality following vaccination with DTPw, caused wide spread panic and concern among parents and physicians (Howson, C. P., 1991, Sato, H., 1999). This negative publicity caused a fall in vaccinations in the UK from 80% to 30%, which later led to two epidemics (Howson, C. P., 1991). In Japan, the negative publicity resulted in temporary suspension of immunization. Although the vaccine was reinstated, vaccination rates were incredibly low, only 20% compared to 70% the previous year (Aoyama, T., 1986, Howson, C. P., 1991, Kimura, M., 1987, Sato, H., 1999, Sato, Y., 1999). Sweden discontinued immunization and in Germany and Italy, rates of vaccination fell dramatically. This decrease in vaccination rates resulted in increased incidence of whooping cough in the aforementioned countries.

Acellular Vaccines

After the controversy surrounding the safety of the pertussis WCV and subsequent epidemic with the decline of its use in Japan in 1979, new acellular vaccines composed of inactivated *B. pertussis* antigens were developed and have been used clinically in Japan since 1981 (Aoyama, T., 1986, Kimura, M., 1987, Sato, H., 1999,

Sato, Y., 1999). Two ACVs were made available in Japan. The Biken or B type is made of inactivated pertussis toxoid and filamentous haemagglutinin in equal amounts and the Takeda or T type consists of more filamentous haemagglutinin than pertussis toxoid and also includes agglutinogens, see below, page 7 (Aoyama, T., 1986, Edwards, K. M., 1995, Sato, H., 1999, Sato, Y., 1999). The two vaccines were examined by a number of laboratory techniques and were found to have reduced toxicity compared with the whole-cell vaccine. Many field studies were conducted in the United States and other countries with the T type Japanese acellular vaccine showing that the adverse effects after being immunized with DTP were much less if it contained the pertussis ACV compared to if it contained the WCV (Mortimer, E. A., 1990, Bernstein, H., 1995). Also, it has been shown that when used as a booster, the ACV induced higher levels of antibodies against PT and FHA (Bernstein, H., 1995).

In 1991 the Advisory Committee on Immunization Practices in the United States licensed the use of DTPa containing pertussis ACV (Takeda or Bilken ACVs) for fourth and fifth doses in children who had a primary course with the WCV (Mortimer, E. A., 1990). Efficacy studies were conducted in countries where the use of the WCV was limited, to determine the efficacy of ACVs administered as the primary vaccination course. Currently a number of different preparations of ACVs have been proven to be safe and effective and were licensed for use by the U.S. FDA as the primary immunization series in 1996 (CDC Health Topics). Four ACVs combined with tetanus and diphtheria are licensed in the United States, and three ACVs in different combinations with diphtheria and tetanus, polio type 1, and Hib are licensed in Canada

(ACS-3). A summary of the licensed acellular vaccines for Canada and the United States is in Table1.1.

The question of whether vaccines against pertussis prevent only the disease, but allow spread of the organism through colonization plagues both the whole-cell and the acellular vaccines. Implementation of the WCV significantly altered the morbidity and mortality of pertussis but the intervals between epidemic cycles have not changed (Fine, P. E. 1988, [illegible]). This suggests that the WCV controlled the disease, but not the circulation of the organism (Black, S., 1997). Although it is too early to predict how the epidemiology of pertussis will change with the institution of the ACV, Japanese researchers suggest that an acellular vaccine composed of pertussis toxoid only, or pertussis toxoid and FHA is sufficient to control pertussis, the disease, similar to what was done with diphtheria (Sato, H., 1999, Sato, Y., 1999). The incorporation of more antigens may lead to not only control of the disease, but control of the spread of *B. pertussis*, as well.

Virulence Factors

B. pertussis produces a number of different virulence factors. A variety of cell-surface molecules are thought to be putative adhesions that adhere to ciliated respiratory cells during the infectious process. *B. pertussis* also produces toxins that are thought to contribute to the clinical disease process. Still, the pathogenesis of pertussis is poorly understood. It is unknown which of the virulence factors are the most important, or if they are all important. The original WCV got around this because all antigens and

virulence factors were included since it was whole-killed *B. pertussis* cells. The problem with the new ACV is deciding which antigens to include in the final formulation.

Adhesions

Fimbriae

B. pertussis fimbriae mediate attachment to eukaryotic cells. Fimbriae are thought to be required for colonization of the respiratory tract, with recent evidence showing they play a role in adherence to the laryngeal mucosa only (van den Berg, B., 1999). Fimbriae most likely are required to sustain that interaction with hosT-cells later in the infection (Hewlett, H., 1997). They are composed of a major subunit, Fim 2 or Fim 3, and a minor subunit Fim D. Fim D is on the fimbrial tip and is found to bind the integrin very late antigen (VLA-5) and heparin sulfate, which are found ubiquitously in the respiratory tract. There is broad agreement that Fim 2 is synonymous with AGG 2 (van den Berg, B., 1999, Hewlett, H., 1997, Kerr, J., 2000). AGG 2 is one of the three major surface agglutinogens that are responsible for the three serotypes of *B. pertussis.* Some think that AGG 3 is synonymous with Fim 3 (van den Berg, B., 1999, Hewlett, H., 1997, Kerr, J., 2000), but this is controversial because type 1,3 cells are nonfimbriate and AGG 3 is located on the cell wall (Kerr, J., 2000). It is more likely that AGG 1 is synonymous with Fim 3 (Woods, M., 1991). Fimbriae are incorporated into some of the new ACVs. A five component ACV containing both FIMs seems more efficacious than ones with just 1 fimbrial type, supporting the contention that both FIM types are needed to protect against colonization in mild disease (Olin, P., 1997).

P. 69 Pertactin

Pertactin, or PRN is a nonfimbrial OMP (Brennan, M. J., 1988), previously known as P. 69 or 69K protein based on its mobility in SDS-PAGE. Based on its sequence it is predicted to have a 60.4 kDa amino-terminal external domain of a 93 kDa precursor polypeptide (Charles, I. G., 1989). The remaining 30 kDa carboxy-terminal domain is located within the outer membrane and is required for the correct localization of pertactin on the cell surface (Emsley, P., 1996). Pertactin is also an agglutinogen and although controversial, is thought to be synonymous with AGG 3 the nonfimbrial surface protein (Brennan, M. J., 1988). Pertactin has been demonstrated to be a protective antigen in mice (Shahin, R. D, 1990), with B-cell epitopes and possibly T-cell epitopes (Charles, I. G., 1989, 1991, Leininger, E., 1991). Pertactin mediates attachment via an Arg-Gly-Asp (RGD) motif, which is thought to bind integrin (Emsley, P., 1996, Leininger, E., 1991). This RGD motif is followed by a series of $(GGXXP)_5$ repeats. A second series of different repeats, (PQP), are found in the carboxy terminal loop that is thought to be the B-cell epitopes (Charles, I. G., 1991, Emsley, P., 1996). Pertactin is found in most ACV formulations that contain 3 or more components.

Filamentous Haemagglutinin

Filamentous haemagglutinin is a filamentous rod-like structure that is first synthesized as a 367-kDa precursor, which is processed to a 220-kDa structure (Piatti, G., 1999). Some FHA is loosely associated with the outer membrane, while the majority is secreted. FHA appears to create a bridge between the cilia and the bacterial surface (Piatti, G., 1999). FHA has at least three different binding sites (van den Berg, B., 1999),

one that binds to glycosaminoglycans, one to carbohydrates, and an RGD motif that has been shown to interact with macrophages via the CR3 integrin. In a recent study by van der Berg *et al.*, it was found that FHA is the only molecule involved in adherence of *B. pertussis* to human bronchial epithelial cells and is a major factor in *B. pertussis* colonization of the entire respiratory tract (van den Berg, B., 1999). When FHA binds CR3 receptor on macrophages it prevents diapedesis. The bacterium is phagocytosed, avoiding a fatal oxidative burst that then allows the bacteria to survive inside the macrophage and multiply intracellularly (Kerr, J., 2000). Immunization with FHA is protective against *B. pertussis* colonization in mice (Piatti, G., 2000), and immunodominant B and T-cell epitopes have been demonstrated. It has therefore been incorporated into most ACVs.

Toxins

Pertussis Toxin

Pertussis toxin is perhaps the most important virulence factor in *B. pertussis*. Pertussis toxoid is included in every formulation of ACV and there is even an ACV of pertussis toxoid alone. PT mutants are avirulent in mice as shown by Alison Weiss (Weiss, A., 1983). Pertussis toxin is an NAD-dependent ADP-ribosyltransferase. The molecular mode of action of pertussis toxin is known to be the transfer of ADP ribose to GTP-binding proteins involved in signal transduction from cell surface to intracellular mediators (Peppoloni, S., 1995), which causes deregulation of the target cell (Mooi, F., 1988). PT is a hexameric AB toxin consisting of five non-covalently linked subunits, S1,

S2, S3, S4, and S5, present in a 1:1:2:1 ratio (Locht, C., 1986). The S1 subunit is the A or the enzymatic ribosyltransferase portion of the AB toxin. The remaining subunits form the B or binding unit that binds to the receptors on the cell surface of eukaryotic cells and facilitates the translocation of the S1 subunit across the cell membrane. Pertussis toxin is known to cause a number of effects *in vitro* and in animals (Williamson, P., 1999), but evidence for these actions in humans, or to the clinical presentation of whooping cough is yet to be elucidated (Hewlett, E., 1999, Kerr, J., 2000). Lymphocytosis, one of effects caused by pertussis toxin is seen in animal models as well as experimentally in humans. It is thought pertussis toxin causes this phenomenon in humans, as evidence gathered from humans injected with pertussis toxin to induce insulin secretion is consistent with this (Hewlett, E., 1999, Kerr, J., 2000).

The clinically-relevant sites of pertussis toxin action and its actual role in the disease are unknown, but some still believe that pertussis toxin is solely responsible for the clinical syndrome of pertussis (Hewlett, E., 1999, Pittman, M., 1979). The symptoms that persist in humans long after the organism has been cleared are thought to be caused by the long-lasting systemic effects of pertussis toxin (Hewlett, E., 1999). Biological effects of pertussis toxin have been shown to persist in humans for 1-2 months after intravenous (IV) injection (Black, S., 1997). Pertussis toxin possibly interferes with normal cough control mechanisms, but this is disputed (Hewlett, E., 1999, Kerr, J., 2000). *B. parapertussis* has been shown to cause the typical symptoms of pertussis even though it does not express pertussis toxin (Black, S., 1997, Hewlett, E., 1999).

Adenylate Cyclase

Adenylate cyclase (CyaA) is a major toxin produced by *B. pertussis*. Mutants deficient in CyaA are avirulent in an infant mouse model (Weiss, A., 1983). CyaA exhibits unique properties when it is secreted by *B. pertussis*. It enters eukaryotic cells and is activated by eukaryotic calmodulin. It then catalyses high-level synthesis of cAMP that alters the cell physiology (Hanski, E., 1989, Kerr, J., 2000). *B. pertussis* CyaA adenylate cyclase (AC) activity has been shown to be greater than 10^6 pmol cAMP/min/mg of protein and to have a k_{cat} of 2000 s^{-1} after activation by calmodulin (Hewlett, E., 1988, Landant, D., 1999). This high level of cAMP can impair the ability of PMN and macrophage chemotaxis, phagocytosis, and oxidative killing of bacteria (Hanski, E., 1989). Recently, it has been shown that CyaA induces apoptosis in macrophages and that the bacterium does not need to be internalized for this to occur (Gueirard, P., 1998, Khelef, N., 1995). CyaA also has hemolytic activity (Glaser, P., 1988). The AC activity is located in the first 400 amino acids, whereas the remaining C-terminal 1306 residues are responsible for hemolytic activity (Lee, S-J., 1999). Both functions work independently of each other.

Human antibodies against CyaA are found after natural infections and after immunization with WCV (Arciniega J. L., 1991, Betsou, F., 1995, Farfel, Z., 1990, Gusio, N., 1993,). Using the intranasal infection model, antibodies against adenylate cyclase were present in mice infected with *B. pertussis* (Betsou, F., 1995). Nonetheless, CyaA has not been included in any of the acellular vaccine formulations. The conflicting evidence about where the protective epitopes are located on the toxin and the difficulty in

purifying the toxin at the time when acellular vaccines were being formulated excluded CyaA from being considered as one of the components (Lee, S-.J., 1999).

Other Outer Membrane Proteins

Porin

Virulent and avirulent *B. pertussis* produce a 40K major outer membrane porin protein. OMP40 (Outer Membrane Protein 40), or major porin, forms smaller anionic selective channels than any porin studied to date, with an average single-channel conductance of 0.56 ns compared to 2.1 ns for the *E. coli* OmpF porin (Armstrong, S. K., 1986). It has a low single channel conductance, a strong ion preference, and a binding site in the channel (Armstrong, S. K., 1986). It is the most abundant protein of the surface of *B. pertussis* (Parker, C., 1988). Using EM, it was demonstrated that *B. pertussis* are covered with a crystalline surface lattice (Kessel, M., 1988). The porin protein forms this crystalline lattice. Computer imaging revealed trimeric channel-like structures. This implies that during normal growth, porin is functional in this crystalline state (Kessel, M., 1988). Interestingly, it was observed that virulent strains examined contained the same amount of the protein as do avirulent strains, but none of the virulent bacteria exhibited the crystalline ordering (Kessel, M., 1988). Antibodies against porin are found in human sera after WCV immunization (Li, Z. M., 1991). In patients recovering from whooping cough, porin was found to produce a strong serum antibody response (Anwar, H., 1991). Porin's importance as a protective antigen is not known.

Acellular Vaccine versus Whole-cell vaccine: Efficacy

As mentioned previously, there have been several ACV efficacy trials taking place in countries that had poor coverage with the WCV, to determine which acellular formulations should be licensed for use as the primary vaccination course. Thirteen candidate acellular vaccines were compared against one another and against one WCV lot (Edwards, K. M., 1995). Of these thirteen, seven were evaluated in a number of efficacy trials that were completed in 1994 and 1995. Along with these seven, a pertussis toxoid ACV candidate was also evaluated for efficacy at the same time. The result of these trials was the licensing of ACVs for use in Canada and the United States as the primary vaccination course. The different ACVs evaluated in the efficacy trials and their components are shown in Table 1.2. The following is a summary of those efficacy trials.

Two trials were conducted in two cities in Sweden. A prospective cohort double blind study was completed in Stockholm where two DTPa containing a 2-component ACV by SmithKline-Beecham or a 5-component ACV by Connaught Canada, were compared to the DTPw by Connaught (US) and DT as a control (Gustafsson, L., 1996, Cherry, J. 1997). The WHO primary case definition of pertussis used in these trials was: an illness with paroxysmal coughing of ≥ 21 days and either culture-confirmed infection with *B. pertussis* or serologic evidence of infection. Based on this definition, the efficacy of the five-component ACV was 85%, the two-component vaccine had an efficacy of 59% while the Connaught (US) WCV had a poor efficacy of 48%. Changing the case definition to include all coughing illnesses with coughing duration greater than 1 day, the efficacy of the five-component remained high at 78% while the efficacy of the two-component and the WCV remained low at 42% and 41% respectively. The other

prospective double blind study performed in Göteberg, Sweden compared a DTPa containing pertussis toxoid only to DT with no DTPw as a control. This trial also used a different immunization schedule than the other study (Trollfors, B., 1995), but the same WHO case definition. Efficacy of the pertussis toxoid was found to be 71%, but when all coughing illness with greater then 7 day duration was included in the definition, efficacy dropped to 53%.

Three studies were carried out in Germany in three cities. A prospective double blind study was done comparing an ACV four-component DTP vaccine from Wyeth Lederle to a DTPw from Lederle (Cherry, J., 1997). Using a slightly modified WHO case definition than the other studies, efficacies of 82% for the DTPa and 91% for the DTPw were found. Schmitt *et al.* did a prospective household contact study comparing SmithKline-Beecham, a three-component acellular DTP, to the Behring DTPw and DT (Schmitt, H.-J., 1996, Cherry, J., 1997). Using the WHO case definition, the ACV had an efficacy of 89% while the WCV had an efficacy of 97%. One other case control study was done, comparing the two-component US Connaught ACV against the standard German DTPw and DT (Cherry, J., 1997). The efficacy of the ACV was found to be 82%, while the efficacy of the DTPw was 96%. In this study, observer bias may have affected interpretation of data that included mild pertussis, making a less effective vaccine appear more effective than it really was.

The trial conducted in Italy by Greco *et al.* was a prospective double blind study comparing a three component ACV from SmithKline Beecham and a three-component from Chiron-Biocine against the Connaught U.S. DTPw and DT (Greco, D., 1996). Using the WHO case definition, the efficacy of the two ACVs were 84% with the WCV

performing poorly with an efficacy of only 36%. When the primary case definition included all illnesses with a cough ≥ 7 days, efficacy rates of 71, 71, and 23% were reported for the two three-component, and whole-cell vaccines respectively.

The last trial was conducted in Senegal comparing a Pasteur-Merieux two-component ACV to the WCV from Pasteur-Merieux (Cherry, J., 1997.). The ACV was found to have an efficacy of 86% while the WCV had an efficacy of 58%, using the WHO case definition. These studies varied in type and number of vaccines, design, case definition, and laboratory method used to confirm the diagnosis of pertussis, which makes it difficult to directly compare any of the trials with each other. Even so, based on prevention of lab-confirmed pertussis with a cough ≥7 days, it can be seen that as the number of components in the ACV increase, so do the efficacies (Cherry, J. D. 1997).

The trial in Italy and the trial conducted by Gustafsson in Sweden showed high efficacies for two three-component ACVs and a five-component ACV and poor efficacies for the US-licensed WCV and a two-component ACV (Gustafsson, L., 1996). Some reviews of the trials concluded that the ACV, regardless of their composition, all had similar efficacies, but were all less effective than the best WCV vaccine. One reviewer, however, suggested that protection increases as the number of components in the ACV increases, especially against mild disease (Potkin, S., 1997). In response to these criticisms, a randomized, double-blind trial was conducted in Sweden comparing the two-component ACV from SmithKline-Beecham, the three-component ACV from Chiron, and the five-component ACV from Connaught Canada, to the UK WCV (Olin, P., 1997). They found that the five-component and three-component ACVs and the UK WCV had similar efficacies against culture-confirmed pertussis with 21 days of cough. The three-

component ACV was less efficacious (71%) than the five-component (79%) and WCV (85%) against culture-confirmed pertussis including all duration of coughing cases. They also found that the two-component ACV was much less protective than the three and five component vaccines. The relative risk of pertussis for ages five to twelve months for the three and five-components vaccines is less than 20% of the relative risk for the two component vaccine. These results confirmed the results of the previous trials (Greco, D., 1996, Gustafsson, L., 1996). It can be concluded that the efficacy of the acellular vaccines depends on the number of components and that different WCV have different efficacies.

Acellular Vaccines versus Whole-cell Vaccines: Reactogenicity

One of the main issues with the WCV, other than efficacy is safety. The WCVs cause adverse reactions in children and they cannot be used as boosters in adolescents and adults because of their reactogenicity. Concerns about safety causes immunization rates to fall, which can contribute to a decrease in efficacy and an increase in the incidence of pertussis disease as discussed earlier with respect to the UK and Japan.

Vaccination with DTPw is associated with local reactions like redness, swelling, and tenderness at the injection site. It is also associated with systemic reactions like fever, drowsiness, fretfulness, vomiting or diarrhea, and more serious, inconsolable crying, occasional persistent screaming, febrile convulsions, excessive somnolence, hypotonic-hyporesponsive episodes (a reaction where the child may loose sensory awareness, become unresponsive or hyporesponsive, loose consciousness accompanied by pallor and muscle hypotonicity or decreased muscle tone) and other rare neurological

events (Boughton, C., 1996, Walker, E., 1988, Howson, C. P., 1991). Despite numerous studies, there is no clear evidence that links DTPw vaccination with neurological damage or encephalopathy (Cherry, J., 1993, Rutledge, 1986).

The efficacy trials for the ACVs included protocols to monitor children for adverse reactions. In all of the trials, the systemic adverse events were much lower for children immunized with ACV than with the WCV (Aoyama, T., 1986, 1996, Cherry, J., 1997, Ciofi, degli Atti, 1999, Decker, M., 1996, Halperin, S., 1999, Olin, P., 1997, Pichichero, M., 1996, Patel, S., 1996). It was also found that the injection site or local reactions seemed to increase as the number of doses increased. Local reactions were more common when the same product was given multiple times whether it was DTPa or DTPw (Halperin, S., 1999). It was suggested by Cherry *et al.* that the decrease in the severe adverse reactions was due to the fact that the endotoxin is not present in ACV preparations (Cherry, J., 1997).

Acellular Vaccine versus Whole-cell Vaccine: Immunogenicity

WCVs induce antibodies to PRN, FHA, PT, FIM, and CyaA (Grimpel, E., 1996,). Antibody titers varied with different WCVs (Baker, J., 1992). The antibody titer also varied depending on whether or not the DTPw was administered with IPV or OPV (Baker, J., 1992). One consistency was that anti-FIM antibodies produced the highest titers, while the anti-PT antibodies stimulated the lowest (Olin, P., 1997). Antibody titers induced by a WCV formulation is shown in Table 1.3. Antibody titers, especially anti-PT, PRN, FIM, tended to decrease rapidly after the fourth injection in the immunization series (Miller, E., 1997). Anti-PT antibodies started to decrease a few months after

immunization, while anti-PRN antibodies and antibodies raised against the agglutinogens started to decline 2 years after immunization (Grimpel , E., 1996, van Savage, J., 1990).

All of the ACVs in the efficacy trials were immunogenic with regards to antibodies (Aoyama, T., 1986, 1996, Cherry, J., 1997, Ciofi, degli Atti, 1999, Decker, M., 1996, Halperin, S., 1999, Olin, P., 1997, Pichichero, M., 1996, Patel, S., 1996). Immunogenicity of the ACVs seemed to depend on the antigen concentration, derivation, and formulation, especially for PT (Decker, M., 1996). All other antigens showed good correlation between concentrations and immunogenicity (Wirsing von König, C. H., 1996), except for FHA. Some vaccine formulations produced lower levels of antibody even though their FHA concentrations were high (Cherry, J., 1997, Wirsing von König, C. H., 1996). Table 1.3 summarizes immunogenicity of some of the ACVs used in the efficacy trials. The ACVs tend to induce higher anti-PT, FHA, and PRN titers in children, than did DTPw (Miller, E., 1997, Guuliano, M., 1998, Halperin, S., 1999, Wirsing von König, C. H., 1996). Overall, the ACVs stimulate better antibody titers than WCVs.

Serological Correlates and Immunity

Serological correlation to immunity to *B. pertussis* is controversial. Even though antibody titers rise after immunization, they do not necessarily correlate with protection (Greco, D., 1996, Gustafsson, L., 1996, Patel, S., 1996, Schmitt, H.-J., 1996). *B. pertussis* antigens have been shown to be protective in mice, but when translated to humans, key antibodies and their protective titers are not known (Poland, G. A., 1996). Many investigators have tried to demonstrate serological correlation with immunity.

Recently, Cherry *et al.* and Storsaeter *et al.* concluded, after looking at data from the acellular trials and from WCV recipients, that high levels of anti-PRN were associated with less susceptibility to disease (Cherry, J., 1998, Storsaeter, J., 1998). But, the general consensus among the scientific community seems to be that no clear correlation between antibody titers and immunity was found in any of the trials (Hewlett, E., 1997, 1998).

Cell-Mediated Immunity

B. pertussis has been considered an exclusively extracellular pathogen and a toxin mediated disease (Cahill, E. S., 1995). Traditionally, research on protection has been focused on the role of the humoral or antibody responses because antigen-specific antibodies are induced in humans and rodents following infection and after immunization (Winsnes, R., 1988). Early animal models of pertussis pathogenesis and immunity all relied on antibody titers as the predictor of protection (Griffiths, E., 1988, Sato, Y., 1988).

B. pertussis may have an intracellular phase in macrophages (Ewanowich, C., 1989, Zepp, F., 1996) in which case cell-mediated immunity could be required for protection. There are recent data showing that cell-mediated immunity plays an important role in recovery and protection against pertussis and that it is a key element in the immunity to *B. pertussis* infections (Ausiello, C., 1997, 1999, Cassone, A., 1997, Mahon, B. 1996, Mills, K., 1993, Redhead, K., 1993, Ryan, M., 1998). Animal studies are conflicting and have failed to resolve what role CMI plays in protective immunity. Some studies describe a Th1 response after WCV and ACV (Cahill, E., 1995), while others found that a Th2 profile was seen after ACV, and only after natural infection or

WCV immunization was the Th1 response seen (Mahon, B. P., 1996, Rehead, K., 1993). It has been suggested by Rehead *et al.* that the type of immune response is dependent on what antigens are in the vaccine and how that antigen is presented (Rehead, K., 1993).

Zepp *et al.* found that CMI was induced in children after immunization with ACV (Zepp, F., 1996), while others have found that WCV induces a Th1 response, and ACV induces a heterogeneous Th1/Th2 or Th0 response (Ausiello, C., 1997, 1999, Ryan, M., 1998, Mills, K., 1999). Taking all of this evidence together, the mechanisms of protection against *B. pertussis* are a complex combination of the two arms of immunity. Indeed, from a study by Mills *et al.*, it was found that *B. pertussis* has multiple protective antigens, and that antibody and T-cell responses against a number of antigens may have an additive effect in protection (Mills, K. H. G., 1998). In mice, high antibody levels appear to be important in limiting the infection and disease by preventing initial adherence to ciliated epithelial cells with an early rapid clearance of bacteria and the CMI seems necessary to completely eliminate the bacteria from its putative intracellular compartments (Mills, K. H. G., 1998, Redhead, K., 1993).

The Changing Epidemiology of Pertussis

The number of cases of pertussis has been increasing since the early 1980's in countries that have had good vaccine coverage with the WCV (Black, S., 1997, Bass, J. W., 1994, De Serres, G., 1995). In 1993 in the U.S., there was an 82% increase in the incidence of pertussis compared to the year before, and the highest incidence of pertussis since 1967 occurred in 1996 (Mahon, C. R., 1999). In Canada, the number of cases

increased to 8030 in 1990 from 2440 the year before. In 1995, the number of cases of pertussis was 10 151 (Health Canada).

A number of factors can be considered as the cause of this recent resurgence. As mentioned earlier, the WCV and the newer ACVs do not afford 100% protection. Disease incidence of pertussis rises and falls in a regular cyclical pattern every 3-4 years. Since the institution of vaccination, this cyclical pattern has not changed or lengthened as would be predicted (Fine, P. E., 1988, 1993), suggesting that vaccination controls disease but not the infection (Black, S., 1997). Also, vaccine-induced immunity, humoral and cell-mediated seems to wane after 6-12 years (Grimpel, E., 1996, Tran Minh, N. N., 1998).

Pertussis in Adolescents and Adults

There has been a marked increase in asymptomatic infections, especially in adults and adolescents, since the early 1980s (Mahon, C. R., 1999). These asymptomatic infections seem to serve as a reservoir for *B. pertussis* infections. Waning immunity probably plays a role in the increased incidence of pertussis in adolescents and adults, but cannot explain the sudden increase, since waning immunity has always been an issue with pertussis immunization. In the United States, the greatest increase in reported cases has been in individuals aged 10-19 years (Guris, D., 1997, 1999). The incidence in adults has also increased in Germany and Canada (Health Canada, Schmitt-Grohé, S., 1995). In most cases of infant hospitalized pertussis, adults and adolescents were the primary cases and transmitters of the disease (Mahon, C. R., 1999, Schmitt-Grohé, S., 1995). In Canada, an acellular pertussis vaccine combined with tetanus and diphtheria

(ADACEL™ manufactured by Aventis Pasteur Limited) has been licensed for use as a booster in adolescents and adults aged 12 to 54 years of age (ACS-1). The National Advisory Committee on Immunization has set up guidelines for its use.

Vaccine-Induced Evolution of Pertussis Virulence Factors

A third factor in the recent resurgence of pertussis is the possibility of vaccine-induced evolution of certain *B. pertussis* virulence factors resulting in antigenically distinct strains from those used in the WCV and ACV vaccines. This was first demonstrated in The Netherlands. Strains isolated in the period from 1949 to 1996 had their sequence of the S1 subunit of pertussis toxin and pertactin analyzed, which revealed polymorphisms in distinct regions in each of these proteins (Mooi, F., 1998). In the S1 subunit, the polymorphisms occurred in areas that are thought to be T-cell epitopes (Mooi, F., 1998, Peppoloni,S., 1995). All the mutations observed in the S1 subunit were single base changes that resulted in amino acid substitutions (Mooi, F., 1998). Three different S1 variants were found in the isolates, S1A, S1B, and S1D, which are now referred to as *ptxS1*A, *ptxS1*B, and *ptxS1*D respectively (Mooi, F., 2000). *PtxS1*A was considered a "new" type since it only appeared in the late 1970s, early 1980s, and from the period of 1990 to 1996, it was found in 80% of the strains being isolated. *PtxS1*B and *ptxS1*D were found to be the types of the strains used for the two Dutch whole-cell vaccines (Mooi, F., 1998). Of the three time periods looked at by Mooi *et al.*; 1949 to 1954, 1978 to 1985, and 1990 to 1996, *ptxS1*D was only isolated between 1949 and 1954. *PtxS1*B was isolated in all three periods, but its frequency decreased over time and

represented 58% 20%, and 12% of the strains in the respective time periods (Mooi, F., 1998).

The polymorphisms in pertactin were observed in two regions of its gene structure (Mooi, F., 1998). Region 1 is comprised an RGD motif followed by a series of GGxxP amino acid repeats, and Region 2 is comprised of PQP repeats. Originally, the focus was on Region 1 since all of the differences observed were confined to this area. In subsequent publications, Region 2 was included in the designation of the different variants (Mooi, F., 1998, 2000), because this area of pertactin is thought to be a B-cell epitope (Charles, I. G., 1991, Mooi, F., 1998). The mutations observed in the pertactin variants coded for amino acid substitutions and/or deletions, or additions of the GGxxP repeats (Mooi, F., 1998, see Figure 6.1 Chapter IV). Three pertactin variants were found originally named P.69 A, P.69B and P.69C. These are now referred to as *prn*1, *prn*2, and *prn*3 respectively (Mooi, F., 2000). *Prn*1 was the pertactin type found in the two strains used for the Dutch whole-cell vaccine, and during the period of 1949 to 1980, 100% of the strains isolated in The Netherlands had *prn*1. Beginning in 1981, *prn*2 and *prn*3 appeared and slowly began to increase until they dominated 90% of the strains being isolated from 1990 to 1996 (Mooi, F., 1998).

The circulating strains in The Netherlands were gradually replaced with antigenic types different from those found in the vaccine strains (Mooi, F., 1998, van Loo, I. H., M., 1999). There have been similar findings reported in Finland, Italy, United States, and Canada (Mooi, F., 1999, Mastrantonio, P., 1999, Peppler, M. S.). A decline in vaccine efficacy due to the *B. pertussis* becoming antigenically distinct from the vaccine strains

may possibly be one of the factors that is contributing to the increase in the incidence of pertussis.

Experimental Design and Purpose of Research

The goal of this research was to determine if strains from Alberta and Québec exhibited antigenic variants in pertactin and the S1 subunit of pertussis toxin, similar to the ones found in The Netherlands, as described above. Strains isolated between 1985 and 1994, and a few from 1995, from the two provinces were examined. Eight initial strains, BpeXba001, BpeXba003, BpeXba008, BpeXba011, CCL-1, CCL-2, CCL-3, and CCL-4 were chosen to have Region 1 in their *prn* gene sequenced and the entire coding region of their ptxS1 genes sequenced, looking for the same type of polymorphisms that were found in The Netherlands by Mooi *et al.* (Mooi, F., 1998). BpeXba001 was the most common pulsed-field type isolated between 1985 and 1994 in Alberta and Québec. BpeXba003 was the most predominantly isolated pulsed-field type in Québec, and BpeXba008 started to dominate the strains being isolated in Alberta in 1994. BpeXba011 was chosen because it was only found in Alberta. The CCL strains were blinded isolates from Connaught Canada (now Aventis Pasteur), two of which were the strains used for the Canadian whole-cell vaccine. The fingerprint of CCL-1 is consistent with tits being the mouse challenge strain 18323, which has a number of unique characteristics that are not typical of *B. pertussis* clinical strains. CCL-2, CCL-3, and CCL-4 were all candidates for the vaccine strains. The sequences of the eight strains showed that pertactin and S1 pertussis toxin variants did exist in the *B. pertussis* population in Alberta and Québec. The pertactin types and S1 pertussis toxin types of these eight strains were then used to

establish the protocol for single strand conformation polymorphism (SSCP), the technique that was used to screen the remainder of the top thirty most common pulsed-field types found in Alberta and Québec.

Two other antigens produced by *B. pertussis*, adenylate cyclase and major porin, were examined for antigenic variation. Adenylate cyclase is an important virulence factor of *B. pertussis*, which was present in the WCV, but was not chosen to be in any of the ACV formulations. Major porin was selected because it is not regulated by the *Bordetella virulence gene (bvg)*. Antigenic variation in adenylate cyclase and major porin was screened for in seven strains, BpeXba001, BpeXba002 the most common isolate in Alberta between 1985-1994, BpeXba008, CCL-1, CCL-2, CCL-3, and CCL-4. The SSCP technique developed to screen for pertactin and pertussis toxin variants was used to screen the seven strains for variants in adenylate cyclase and major porin.

The main epidemiological tool used in *B. pertussis* epidemiology in Alberta, Québec, and the rest of Canada had been pulsed-field gel electrophoresis (PFGE). Genomic DNA set into an agarose plug is digested with a restriction enzyme, usually Xba I. This digested DNA is then run out on an agarose gel while the current is pulsed at different angles resulting in a pattern of bands. *B. pertussis* strains can be assigned a "pulsed-field" type according to their resultant Xba I digestion pattern and grouped. It has been suggested by Stibitz, S, *et al.*, that the different patterns seen are a result of chromosomal rearrangements (Musser, J., 1986, Stibitz, S., 1997, 1999).

During the ten-year period of 1985-1994 in Alberta and Québec, 102 different pulsed-field types showed variations in their frequency of isolation, with certain ones dominating while others disappearing as quickly as they appeared (Peppler, M. S.,

manuscript submitted). This implied that there might be a selective advantage for some PFGE types.

The antigenic typing described in this thesis is another tool that can be used in the epidemiology of *B. pertussis* in conjunction with PFGE. Together, the two techniques may answer questions pertaining to the resurgence of pertussis in Canada.

Acellular Vaccines Licensed in Canada			Acellular Vaccines Licensed in the U.S		
Producer	**Vaccine**	**Components**	**Producer**	**Vaccine**	**Components**
Pasteur Mérieux Connaught Canada (Aventis Pasteur)	*TRIPACEL™ (DTPa)	PT (20 µg) FHA (20 µg) PRN (3 µg) Fimbriae (combined AGG types 2 and 5) (3 µg) Diphtheria (15Lf) Tetanus (5Lf)	Wyeth Lederle	Acel-Imune ®	PT 3.5 mcg/dose FHA 35 mcg/dose PRN 2 mcg/dose AGG 0.8mcg/dose
Wyeth-Ayerst Canada Inc.	Acel-P™ (Pa)	Pertussis antigens (40-60 µg) PT (8%) FHA (86%) PRN (4%) Fimbriae AGG type 2 (2%)	SmithKline Beecham	Infanrix®	PT 25 mcg/dose FHA 25 mcg/dose PRN 25 mcg/dose
	ACEL-IMMUNE ™ (DTPa)	Same as Acel-P™ plus Diphtheria (7.5 Lf) Tetanus (5 Lf)	Aventis-Pasteur	Tripedia®	PT 23 mcg/dose FHA 23 mcg/dose
SmithKline Beecham Pharma Inc.	Infanrix™ (DTPa)	PT (25 µg) FHA (25 µg) PRN (8 µg) Diphtheria (30 IU) (25 Lf) Tetanus (40 IU) (10 Lf)	North American Vaccine	Certiva ®	PT 40 mcg/dose

*QUADRACEL™ (TRIPACEL™ plus polio type 1 (Mahoney), type 2 (M.E.F.1), and type 3 (Saukett)) AND PENTACEL™ (TRIPACEL™ plus Act-HIB® (PRP-T 10 µg) also available.

Table 1.1. Summary of licensed acellular vaccines in Canada (Health Canada website, Vaccine-Preventable Diseases, 1997) and the United States (Centers for Disease Control and Prevention website, Health topics A-Z).

Manufacturer	Abbreviation	Pertussis Toxin (µg/dose)	Filamentous Haemagglutinin (µg/dose)	Pertactin (µg/dose)	Fimbriae (µg/dose)
Amvax	AM-1	40	0	0	0
Connaught-US (Connaught/Biken)	CU-2	23.4	23.4	0	0
Pasteur-Merieux	PM-2	25	25	0	0
Smith-Kline Beecham	SKB-2	25	25	0	0
Chiron-Biocin	CB-3	5	2.5	2.5	0
SmithKline Beecham	SKB-3	25	25	8	0
Wyeth-Lederle (Lederle/Takeda)	WL-4	3.5	35	2	0.8
Connaught-Canada (Aventis Pasteur)	CC-5	10	5	3	5

Table 1.2. The contents of the acellular vaccines evaluated in the seven efficacy trials.

Vaccine	No. of antigens	PT µg/dose	FHA µg/dose	PRN µg/dose	FIM µg/dose	PT Titer	FHA Titer	PRN Titer	FIM Titer
Swiss Serum and Vaccine Institute	1	50	0	0	0	99	1	3	2
SmithKline Beecham	2	25	25	0	0	104	110	3	2
Connaught (US)/Biken	2	23.4	23.4	0	0	127	84	3	2
Pasteur Merieux	2	25	25	0	0	68	143	3	2
SmithKline Beecham	3	25	25	8	0	54	103	185	2
Biocine	3	5	2.5	2.5	0	99	21	65	2
Lederle/Takeda	4	3.5	35	2	0.8	14	49	54	51
Connaught Canada (Aventis Pasteur)	4	10	5	3	5	36	37	114	240
WCV (Lederle)	-	-	-	-	-	67	3	63	191

PT-pertussis toxin
FHA-filamentous haemagglutinin
PRN-pertactin
FIM-fimbriae

Table 1.3. Examples of the vaccines used in efficacy trials, their contents, and resulting mean antibody titers in ELISA units (Cherry, J., 1996). Antibody titers for pertussis-toxin depended on antigen concentration, antigen formulation, and derivation. FHA antibody titers varied, some correlated with concentration while others produced levels that were lower than some vaccines that contained less FHA. Pertactin antibody titers correlated well with concentration. Fimbriae also correlated well with concentration. The WCVs shown here produce less anti-PT, PRN, and FHA antibodies compared to some of the ACVs.

CHAPTER II

MATERIALS AND METHODS

2.1. Bacteria

The Alberta *Bordetella pertussis* strains isolated between 1985 and 1994 were provided by the Provincial Laboratory of Northern Alberta, Edmonton AB, and the Québec *B. pertussis* strains were provided by the Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, PQ. The Alberta strains were frozen in Greave's solution (1.75% bovine serum albumin, 5% sodium glutamate in chemically-pure water) at -70°C, until needed. The desired strains were grown up from frozen stocks on Bordet-Gengou agar (BGA) supplemented with 1% glycerol and 15% sheep from Daylin Laboratories (Calgary, Alberta) blood at a humidified 37°C for 3 to 5 days.

2.2. Isolation of genomic DNA

Genomic DNA was isolated from the bacteria following the protocol of Ausubel, F. M. (1992). The required strains were grown from frozen stocks as stated above. Each strain's growth was then scraped into 5 ml of Stainer-Scholte (SS) broth, in 50 ml tubes. The bacteria were then grown in the broth in a 37°C shaker incubator to stationary phase or from 3 to 5 days. The growth was transferred to a 1.5 ml microcentrifuge tube and spun in a microcentrifuge for 2 minutes. Alternatively, and the preferred method, the broth growth step was skipped and the procedure was done as follows. Growth from a

BGA plate was scraped and suspended directly into a microcentrifuge tube containing 1.0 ml of SS broth. The bacterial suspension was spun in a microcentrifuge for 2 minutes the same as if it were grown in the SS broth. The pellet was resuspended in 567 µl TE (10 mM Tris-buffer, pH 8.0, 1mM EDTA) by repeat pipetting. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K (Boehringer Mannheim) was added, mixed and incubated at 37°C for 1 hour. After incubation, 100 µl of 5M NaCl was added and mixed thoroughly. 80 µl of CTAB/NaCl solution (Ausubel, F. M., 1992) was added, mixed, and incubated at 65°C for 10 minutes. After incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added, the solution was mixed and microcentrifuged for 5 minutes. The supernatant was transferred to a new tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant, the solution was mixed and microcentrifuged for 5 minutes. The supernatant was transferred to a new tube. Isopropanol (0.6 times the volume of the supernatant) was added to the supernatant and mixed gently until DNA precipitated. The precipitate was washed with 70% ethanol, mixed and microcentrifuged for 5 minutes. The ethanol was drained, and the tubes were inverted to dry. The DNA was resuspended in 100 µl TE buffer.

The Québec strains were not stored at -70°C like the Alberta strains, so the genomic DNA for these strains was extracted from pulsed-field plugs. The plug was immersed in 100 µl of TE buffer and frozen at -20°C overnight. It was then thawed and mechanically disrupted with a pipette tip so the agarose was mashed into tiny pieces and released some of the DNA into the TE buffer. When 5 µl of these mixtures were used for PCR, a tiny piece of agarose was always added with the TE buffer.

2.3. Sequencing of the pertactin gene and the S1 subunit of pertussis toxin genes

347 bp of region 1 of the *prn* gene and the entire *S1* gene of pertussis toxin (*ptxS1*) were sequenced for 8 strains: BpeXba001, BpeXba003, BpeXba008, BpeXba011, CCL1, CCL2, CCL3, CCL4.

Using the genomic DNA isolated for the eight strains, region 1 of the *prn* gene was first amplified using primers PF and PR (Table 2.1). The reaction mixture was comprised of the following, adapted from Mooi *et al.*, 1998. The *prn* gene was amplified in 50µl containing 10 µl 10X PCR buffer (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada), 10% dimethyl sulfoxide, 2 mM $MgCl_2$, 0.4 mM each deoxynucleotide, 10 pmol each primer, template DNA, and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada). The reaction mixtures were amplified as follows: 1 step for 3 minutes at 95°C, followed by 30 cycles of 20 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C, and then ending with 1 final step for 7 minutes at 72°C. 5 µl of the reaction mixture was visualized on an ethidium bromide-stained 0.8% agarose (BioRad, Mississauga, ON, Canada) gel to see if the 1427 bp was amplified. The PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Inc., Mississauga, ON, Canada), and sent to the University of Alberta DNA Core Lab (Department of Biochemistry, University of Alberta, Edmonton, AB, Canada) where it was sequenced with an automated sequencer (ABI Prism, model version 2.1.1) using primers PF2 and PR2 (Table 2.1). This resulted in a 347 bp product being sequenced.

The entire S1 coding region for pertussis toxin was amplified using the same eight strains as for the pertactin gene, using the primers S1F and S1R (Table 2.1). The *ptxS1*

gene was amplified according to the procedure of Mooi *et al.*, 1998, in 50 µl containing 10 µl of 10X PCR buffer (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada), 0.1% Triton X-100, 5% dimethyl sulfoxide, 0.4 mM each deoxynucleotide, 1.5 mM $MgCl_2$, 10 pmol of each primer, template DNA, and 2.5 units of *Taq* DNA polymerase (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada). The reaction mixtures were treated as follows: 1 step for 3 minutes at 95°C, followed by 30 cycles of 15 seconds at 95°C, 15 seconds at 59°C, and 1 minute at 72 °C, and then ending with 1 final step for 10 minutes at 72°C. 5 µl of the PCR products were first visualized on 0.8% agarose (BioRad, Burlington, ON, Canada) gel stained with ethidium bromide, and then the PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Inc., Mississauga, ON, Canada). The purified PCR products were then sent to the University of Alberta DNA Core Lab (Department of Biochemistry, University of Alberta, Edmonton, AB, Canada) and sequenced with the same automated sequencer used for the pertactin sequences, with primers S1F and S1R, and S1FM and S1R.

These eight strains were found to have different types of *prn* and *ptxS1* sequences and were subsequently used to establish the single strand conformation polymorphism (SSCP) protocol (Markoff, A., 1997, Oto, M., 1993, Peng, H., 1995).

2.4. Nomenclature

Strains were named and grouped according to their pulsed-field pattern that resulted from Xba I genomic digestions separated by pulsed-field gel electrophoresis (Peppler, M. S.). The strains with the same patterns are grouped and designated by BpeXba (Bpe = *B. pertussis*, Xba = enzyme used) and a 3 digit number assigned

according to how commonly isolated the strain type was in Alberta and Quebec combined during the ten year time period.

The subscript in the names of the pertactin variants, for example *prn*1_1, refers to which part of the gene was sequenced to determine the pertactin type. The subscript 1 means that Region 1 was the only region sequenced, a subscript of 2 would mean that region 2 was the region sequenced, a subscript of 1,2 means that both regions were sequenced, and no subscript means that the entire pertactin gene was sequenced.

The term "old" refers to antigenic variants of pertactin or S1 pertussis toxin, *ptxS1*D, *ptxS1*B, and *prn*1, that were present in vaccine strains and in the strains isolated in the 1930s and 1940s (Cassidy, P.). "New" pertactin and S1 pertussis toxin types are variants, *ptxS1*A, *prn*2, and *prn*3, that started to appear in the late 1970s for pertussis toxin and the early 1980s for pertactin (Cassidy, P.).

Antigenic profiles consist of *prn/ptxS1* types. Strains that have two "old" antigens are considered to have an "old" profile, strains with an "old" and a "new" antigen are considered to have a "transitional" profile, and strains with two "new" antigens are considered to have a "new" profile (Cassidy, P., 2000).

2.5. Single Strand Conformation Polymorphism (SSCP)

2.5.1. Polymerase Chain Reaction (PCR)

Single base changes or additions/deletions of repeats can be visualized using SSCP. Single strands of denatured DNA adopt a conformation based on their sequence. If a single base is changed in a sequence, it can adopt a slightly different conformation as a single strand. The mobility of DNA through a non-denaturing acrylamide gel is based

on the sequence of DNA. Therefore, the different conformations adopted by single stranded wild type and single stranded mutant DNA will have different mobility through the non-denaturing gel.

All the PCR products used for SSCP were between 200 and 350 bp. A computer program designed to help choose appropriate primers called Primer3 Output, was used to choose the PCR-SSCP primers for pertactin, the S1 subunit of pertussis toxin, major porin, and adenylate cyclase. All the primers used are shown in Table 2.1 and Table 2.2. PF2 and PR2 are the primers used for amplification of the pertactin PCR product used for SSCP and S1BF and S1R are the ones used for amplification of the S1 subunit of pertussis toxin PCR product used for SSCP.

The PCR protocols and amplification procedure for pertactin and the S1 subunit of pertussis toxin used for SSCP are the same ones that were used for sequencing, as stated previously. The pertactin PCR protocol and amplification procedure was also used for amplifying adenylate cyclase and major porin for SSCP using the primers in Table 2.1 and Table 2.2. All PCR products were purified by cutting the bands of interest out of a 0.8% agarose gel stained with ethidium bromide. The piece of agarose containing the bands of interest were put into a SpinX tube (Fisher Scientific, Nepean, ON, Canada), made up of a column and a microcentrifuge tube, and spun in a microcentrifuge. The SpinX tubes allows the PCR product and some liquid from the agarose to pass through into the microcentrifuge tube, with the bulk of the agarose remaining in the column. The PCR product was precipitated by adding 1 ml of 95% ethanol to the solution in the microcentrifuge tube, which was then cooled for at least 30 minutes at -20°C. After the

cooling process the solution was spun in a microcentrifuge for 5 minutes. The pellet was resuspended in 40 µl Milli-Q water (double distilled filtered water).

2.5.2. Sample Preparation for SSCP

10 µL of the purified PCR product was mixed with 10 µL of formamide loading buffer (980 ml/L formamide, 10mmol/L EDTA, 0.25 g/L xylene cyanol FF, and 0.25 g/L bromphenol blue) and denatured in a boiling water bath for 4 minutes. The samples were immediately placed in ice and loaded on to the gel.

2.5.3. SSCP

The Mini-Protean II apparatus (BioRad, Mississauga, ON, Canada) was used to cast the acrylamide/agarose composite gels (Peng, H., 1995) in a 1:1 ratio with final concentrations of 10% acrylamide (37:1 acrylamide/ bisacrylamide solution from BioRad, Mississauga, ON, Canada) and 0.25% agarose (0.5% agarose made with 2X TBE). A 20% acrylamide solution was made up to a 5 ml volume and split into two tubes, 2.5 ml each and put into a 30°C water bath. The 0.5% agarose solution in 2X TBE was heated and slightly cooled. The following steps had to be done quickly to prevent the agarose in the solution from hardening. 2.5 ml of the agarose solution was added to the 2.5 ml of acrylamide. The solution was vortexed, then 35 µl of 10% ammonium persulfate (APS) was added and vortexed, then 5 µl of TEMED (Gibco BRL, Canadian Life Technologies Burlington, ON, Canada) was added and vortexed. The solution was transferred to the mold with a Pasteur pipette. The gels were allowed to solidify, and the

samples were loaded. The gels were run at 5 watts for 2.5 hours with Tris-glycine buffer (0.5 M Tris, 50 mM glycine).

2.5.4. Silver Staining

The gels were fixed in about 83 ml (250 ml divided into three) 12% acetic acid with 0.02% formaldehyde for 5 to 18 hours followed by 3 washes with Milli-Q water, 2 minutes each. They were then stained using a silver stain solution for LPS as follows: 5.6 ml NaOH was added to 50.4 ml of Milli-Q water. 4 ml of concentrated ammonium hydroxide was added. With vigorous mixing, 10 ml of 20%w/v silver nitrate was then added to the solution, followed by 230 ml of Milli-Q water. The solution was divided evenly between the gels, which were stained for 30 minutes. After staining, the gels were washed for 30 sec with Milli-Q water and then developed (1 liter of Milli-Q water containing 0.5ml 37% formalin and 50mg citric acid). Developed gels were placed in 7% acetic acid. The gels were photographed and dried using BioGelWrap (BioDesign Inc., Carmel, New York, USA).

2.6. SSCP Analysis With Capillary Electrophoresis

2.6.1. PCR

The different pertactin alleles were amplified using primers PF2 and PR2 labeled with the fluorescent TAMRA dye (University of Calgary DNA Facility, Calgary, Alberta, Canada). The same pertactin PCR protocol and amplification procedure used for sequencing and acrylamide gel SSCP was used to generate the pieces of DNA for capillary electrophoresis. TAMRA (absorbs at 555nm and emits at 580 nm) is the dye

used with the helium neon laser (emits at 543 and 594 nm). The PCR products were purified the same way as the ones used for acrylamide gel SSCP using the SpinX tubes.

2.6.2. Sample Preparation

The purified PCR product, formamide, and 0.3 N NaOH were mixed in a 1:1:2 ratio (SSCP analysis: Chapter 7). This mixture was heated in a boiling water bath or on a hot plate for 4 minutes to denature the sample. The samples were then immediately put on ice.

2.6.3. Capillary Electrophoresis

SSCP analysis with capillary electrophoresis was done on a five capillary machine made by Dr. JianZhong Zhang, in the lab of Dr. Norm Dovichi, Department of Chemistry, University of Alberta, Edmonton, AB, Canada. Dr. Woei Tan helped with the scientific background of the technique and with use of the machine. The five 45 cm capillaries with an outer diameter (OD) of 150 μm and an inner diameter (ID) of 48 μm, were filled with 7% GeneScan polymer (ABI Prism, PE Applied Biosystems, Streetsville, ON, Canada) diluted 1:1 with 1X TBE containing 10% glycerol running buffer for a final concentration of 3.5%. The conditions for the capillary electrophoresis are as follows: first, the samples were introduced into the polymer filled capillaries with an injection voltage of 100 V/cm and an injection time of 40 seconds. The samples were then replaced with the 1X TBE containing 10% glycerol running buffer, and the capillary electrophoresis (CE) voltage was set at 200 V/cm. The samples emerge from the capillaries after about 25-30 minutes.

Primers	Sequence (5′-3′)	Gene	Position
PF*	GCCAATGTCACGGTCCAA	*prn*	649-666
PR*	CGGATTCAGGCGCAACTC	*prn*	2076-2059
PF2	TGGTGCTGCGCGACACCAAC	*prn*	746-766
PR2	GACTGGGCGAGCTCCAAGCT	*prn*	1073-1053
S1F*	TAGGCACCATCAAAACGCAG	*ptxS1*	474-493
S1R*	TCAATTACCGGAGTTGGGCG	*ptxS1*	1350-1330
S1FM*	ACAATGCCGGCCGTATCCTC	*ptxS1*	949-965
S1BF	ATTCCAACGCTCGCTACGTC	*ptxS1*	1090-1109
POR1F	GCATTGCTGCTCTTGTCACT	*Omp40*	406-426
POR1R	TCACCCAGATCTTCCGTACC	*Omp40*	692-672
POR2F	GGTACGGAAGATCTGGGTGA	*Omp40*	673-693
POR2R	TACGACGGGGTCTGGTACAT	*Omp40*	971-951
POR3F	GTCATGTACCAGACCCCGTC	*Omp40*	949-969
POR3R	AGAGCCAGCTTCACGACTTC	*Omp40*	1235-1215
POR4F	GAAGTCGTGAAGCTGGCTCT	*Omp40*	1216-1236
POR4R	GTAGGTGTAGCCCAGCGAGA	*Omp40*	1491-1471
POR5F	AGGCCAACTCGTACATGGTC	*Omp40*	1355-1375
POR5R	AATCGAAGCGGAACTTGC	*Omp40*	1636-1618

* Primers taken from reference Mooi. F., *et al.*, 1998.

Table 2.1. Primers for pertactin, S1 subunit of pertussis toxin, and major porin used in this study and their position in their respective sequences.

	Primers	Sequence (5′-3′)	Gene	Start Position (aa)
Region 1	AC4F	AGAACAATCCTTTCCCGGAG	*CyaA*	302
	AC4R	ACCCCATCAAGGCTGTCATA	*CyaA*	410
	AC5F	CTATGACAGCCTTGATGG	*CyaA*	403
	AC5R	GCCGAATTGCGTCATCAG	*CyaA*	486
	AC1,2F	GCCCTGATGACGCAATTC	*CyaA*	480
	AC1,2R	GACCGTTCCACCTGTCAACT	*CyaA*	580
	AC3,4F	GGATGTCGTTGACCGATGA	*CyaA*	552
	AC3,4R	GTCCAGCTGATCCGCATAGT	*CyaA*	637
	AC5,6F	AATCGCACTATGCGGATCA	*CyaA*	629
	AC5,6R	CCTGCAGGTTTTTCTCGAAG	*CyaA*	745
	AC7F	CGTACTTCGAGAAAAACCTG	*CyaA*	737
	AC7R	GATCTCGACGAATGTGGTG	*CyaA*	869
Region 2	AC8F	AGCG AATTCACCACATTCG	*CyaA*	861
	AC8R	ACATGGCGGTATTGGACATTC	*CyaA*	993
	AC9F	AATGTCCAATACCGCCATG	*CyaA*	987
	AC9R	GTGCACGTTGTACTTCAC	*CyaA*	1092
	AC10F	GAAGTACAACGTGCACCAG	*CyaA*	1087
	AC10R	GTCCTGCAGGAAGATGTCG	*CyaA*	1193
	AC11F	CACGCTGTATGGCGAGGAC	*CyaA*	1179
	AC11R	GATGAGCACATCCTTCATG	*CyaA*	1270
	AC12F	ACGTCATCGGTACGAGCATG	*CyaA*	1259
	AC12R	GTCTGGCTGTAATCGACG	*CyaA*	1357
	AC13F	CGTCGATTACAGCCAGAC	*CyaA*	1351
	AC13BR	TCCTGGAAGAACCAGTCGTC	*CyaA*	1467
	AC14F	GACTGGTTCTTCCAGGATGC	*CyaA*	1462
	AC14R	AGGCCATTGAGGACGTTGGC	*CyaA*	1558
	AC15F	CAACGTCCTCAATGGCCTG	*CyaA*	1542
	AC15R	TACCAGTCGTGCACGGTAAG	*CyaA*	1646
	AC16F	TACCGTGCACGACTGGTATC	*CyaA*	1640
	AC16R	AAGAACGCAAAGAGACGGCG	*CyaA*	1727

Table 2.2. Primers used for amplification of the adenylate cyclase toxin gene and their amino acid start position.

CHAPTER III

ANTIGENIC VARIATION IN THE S1 SUBUNIT OF PERTUSSIS TOXIN AND PERTACTIN

Introduction

There are a number of factors that are thought to be the cause of the recent resurgence of pertussis in countries that have had good coverage with the WCV (Black, S., 1997, Bass, J. W., 1994, De Serres, G., 1995). These factors include less than 100% protection from the vaccines, waning vaccine-induced immunity, increases in pertussis in adults and adolescents, and vaccine-induced antigenic variation in important virulence factors, as explained in Chapter I. Antigenic variation in *B. pertussis* virulence factors was first demonstrated in The Netherlands by Mooi *et al.* and proposed to be the reason for the rise in incidence seen in whooping cough (Mooi, F., 1998, van Loo, 1999). In the original paper, Mooi *et al* sequenced the entire pertactin and pertussis toxin S1 subunit genes and found sequence variations among the strains (Mooi, F., 1998).

For the purpose of this thesis, in order to demonstrate antigenic variation in pertactin and the S1 subunit of pertussis toxin in strains isolated in Alberta and Quebec between 1985 and 1994, it was decided to use SSCP as a screening method. A study was being conducted in our lab using PFGE as way to epidemiologically group *B. pertussis* strains isolated in Alberta and Quebec between 1985-1994. An outbreak of *B. pertussis* occurred during this ten-year period between 1989 and 1991. One hundred and two

different pulsed-field types were demonstrated in the ten-year time period (data not shown). Antigenic typing was done on this group of strains isolated between 1985 and 1994, hoping it would contribute to examining the epidemiology of pertussis in this ten-year period.

SSCP was chosen as the screening technique because it was more cost effective then sequencing pertactin and S1 pertussis toxin for all of the strains. Some other techniques for detecting point mutations and addition/deletion mutations are chemical cleavage of mismatch (CCM) (Cotton, R. G. H., 1988, Verpy, E., 1994), heteroduplex analysis (White, M. B., 1992), denaturing gradient gel electrophoresis (DGGE) (Myers, R., 1987), Rnase cleavage (Myers, R., 1985), and enzymatic cleavage of mismatches (Youil, R., 1995, Marshal, R., 1995).

First, eight strains, BpeXba001, BpeXba003, BpeXba008, BpeXba011, CCL-1, CCL-2, CCL-3, and CCL-4 were chosen to have key regions in pertactin and S1 subunits sequenced. As previously mentioned, BpeXba001 was the most prevalent pulsed-field type isolated in Alberta and Québec combined in the ten-year period, BpeXba002 was the most commonly isolated type in Alberta, BpeXba003 was the most predominantly isolated type in Quebec, and BpeXba008, an emerging pulsed field type, with 66.5 % of 326 strains isolated in Alberta in 1995, and thereafter, between 56-72% (data not shown). The CCL strains are blinded strains from Connaught Canada Labs (now Aventis Pasteur), two of which are the vaccine strains used as the Canadian whole-cell vaccine and as the basis for the ACV. The pertactin and S1 subunit of pertussis toxin of these eight strains were then used to set up the protocol for the SSCP. Once the SSCP procedure was

established, a representative strain from the remaining top thirty pulsed-field types in Alberta and Québec were antigenically typed.

Results and Discussion

For each of the eight strains, a 1427 bp region of the pertactin gene was amplified using primers PF (GCC AAT GTC ACG GTC CAA) and PR (CGG ATT CAG GCG CAA CTC) (Table 3.1). The resulting amplicon includes both of the repeat regions of pertactin that may contain variations. Since Mooi *et al.* found that the variations seemed to be confined to Region 1 (Mooi., F., 1998), this is the region that we focused on for the SSCP. The pertactin amplicons of the eight strains were sequenced using primers PF2 (TGG TGC TGC GCG ACA CCA AC) and PR2 (GAC TGG GCG AGC TCC AAG CT) (Table 3.1). This focused the sequence on an area of 347 bp, which contained repeat Region 1. For the S1 subunit of pertussis toxin, the entire S1 gene was amplified for each of the seven strains using primers S1F (TAG GCA CCA TCA AAA CGC AG), S1FM (ACA ATG CCG GCC GTA TCC TC), and S1R (TCA ATT ACC GGA GTT GGG CG) (Table 3.1). The S1 amplicons were then sequenced to determine if they contained any antigenic sequence changes that would account for antigenic variation. The resulting pertactin sequences of the eight strains are shown in Figure 3.1 and the resulting S1 subunit sequences are shown in Figure 3.2. These strains did indeed show antigenic variation in the S1 subunit of pertussis toxin and pertactin, with their sequences matching the sequences published by Mooi *et al.* shown in Figure 3.3 (Mooi, F., 1998). Based on this comparison, the BpeXba types were assigned a corresponding *prn* and *ptxS1* type. BpeXba001 has a "new" pertactin type $prn2_1$ and a "new" S1 pertussis toxin type

*ptxS1*A; BpeXba003 has an "old" pertactin type *prn*1_1 and an "old" S1 pertussis toxin type *ptxS1*B, BpeXba008 and BpeXba011 have the same "new" antigen types as BpeXba001 (refer to Materials and Methods, section 2.4 Nomenclature).

As previously stated, CCL-1 to 4 are blinded strains (Connaught Canada), two of which are the strains used for the whole-cell vaccine in Canada. CCL-2 and CCL-4 have the same "old" pertactin type *prn*1_1 as BpeXba003, but a different "old" S1 pertussis toxin type, *ptxS1*D. In The Netherlands, *ptxS1*D was only seen in strains isolated in pre-vaccine and immediately post-vaccine years and in one of their vaccine strains (Mooi, F., 1998). CCL-3 has "old" antigens of *prn*$1_1$and *ptxS1*B, which was also found in BpeXba003 and in one of the vaccine strains used in The Netherlands (Mooi, F., 1998). Comparing the antigenic types of CCL-1, CCL-2, and CCL-3 to the antigenic types of strains that are known vaccine strains, any of the three can be candidates for the WCV used in Canada.

CCL-1 had unique pertactin and S1/pertussis toxin types (*prn*6_1/*ptxS1*E), the sequences shown in Figure 3.3, combined with its unique pulsed-field pattern (Peppler, M.) lead us to believe that CCL-1 is 18323, the mouse challenge strain. 18323 is not a typical *B.* pertussis strain. It was chosen as the mouse challenge strain because it possesses virulence for mice not shown by any other strain of *B. pertussis* (Adams, G. J., 1970, Musser, J., 1986). 18323 has shown significant differences in the sequence analysis of its pertussis toxin operon compared to more typical strains of *B. pertussis* (Arico, B. R., 1987). Using multilocus enzyme electrophoretic analysis, it was shown by Musser, *et al.* that 18323 is more closely related to *Bordetella bronchiseptica* than to *B. pertussis* (Musser, J., 1986)

Now that the pertactin and S1 subunit/pertussis toxin of the eight strains were known, we proceeded to work out a protocol for SSCP. In order for SSCP to work, the PCR products run must be small. For pertactin, using primers PF2 and PR2 (Table 3.1) to amplify region 1 resulted in a 347 bp amplicon, suitable to use for SSCP. The S1 subunit of pertussis toxin was amplified using primers S1BF (ATT CCA ACG CTC GCT ACG TC) and S1R (Table 3.1), which resulted in a 257 bp fragment focused on the polymorphisms in region 3 of the S1 subunit where others have shown antigenic variations. First, the amplified pieces were run on an agarose gel srained with ethidium bromide. In order to have a sample containing only the DNA of interest, the correct bands were cut out of the gel and precipitated as described in Chapter II. The purified pertactin and S1 subunit DNA of the eight strains were then run on agarose/acrylamide composite gels with Tris-Glycine running buffer at 5W for two and a half hours. After fixing the gels in an acetic acid/formaldehyde solution, they were silver-stained to visualize the resulting patterns. Usually two bands are present representing the sense and the antisense strands of the denatured DNA. Sometimes only one band appeared, suggesting that the two single strands had the same mobility. The different pertactin types were easily distinguishable from each other. It can be seen in Figure 3.4 that their migration patterns down the gel and the distance between the two bands, if present, are very distinct. For example, just by visual inspection of the gel, it can be seen that BpeXba001, BpeXba003, and CCL-1 have different pertactin types and that BpeXba001 and BpeXba008 have the same type, as do BpeXba003, CCL-2, CCL-3, and CCL-4. The patterns for the different S1 types were subtler than the pertactin patterns (Figure 3.5). All have two bands, but the distance between the two bands, although similar for all the

S1 patterns, allows distinction among types. For example CCL-1, CCL-3 and BpeXba003 can be seen to be similar. If the patterns of these three strains were compared without knowing the sequence of their S1 pertussis toxin genes, CCL-1, CCL-3 and BpeXba003 would be considered to have the same S1 pertussis toxin type. It was still possible to screen the S1 subunit of pertussis toxin for the remaining strains by SSCP, but a lane containing a known *ptxS1* type was run along side the unknown samples as a reference.

The same SSCP protocol was used to screen the top 30 most common pulsed-field types out of 102 types in Alberta and Québec between 1985 and 1994 (Peppler, M. S., manuscript on preparation). These thirty pulsed-field types represent 93% of the strains isolated in the ten-year period. A single representative isolate from each of the top 30 pulsed-field types was chosen to be antigenically typed. Their pertactin and S1 pertussis toxin genes were amplified and their SSCP profiles were compared to the standards tested earlier. Table 3.2 lists the 30 pulsed-field types, their antigenic profiles (*prn/ptxS1* type), and number of isolates in each group.

We found a new pertactin type and using the new nomenclature by Frits Mooi was named *prn*9_1, because it is the ninth pertactin type found world wide. BpeXba023 and BpeXba029 both have *prn*9_1. Its resulting SSCP pattern is shown in Figure 3.6. The *prn*9_1 pattern is similar to the pattern for *prn*1_1, in that the distance between the two bands is the same, but the migration of the pattern down the gel is slower than the pattern for *prn*1_1. The sequence for *prn*9_1 differs from *prn* types 1, 2, and 3 by the addition of extra GGFGP repeats (Figure 3.7).

Another interesting pattern was found with BpeXba030, a pulsed-field type found only in Québec. It is a mixture of *prn*1_1 and *prn*2_1 SSCP patterns (Figure 3.6). When sent for sequencing, the data were inconclusive. To try and resolve this ambiguity, six more BpeXba030 strains were tested for their pertactin types. Two strains had the pertactin type 1_1, one turned out to be pertactin type 2_1 and two had the combination pattern. In order to establish whether a mixture of two strains causes this combined pattern, ten isolated single colony clones from the frozen stock should have their pertactin types determined to see if all of the colonies are producing the same pattern. I was unable to do this in time for this thesis, because we only have access to the DNA from BpeXba030. The frozen strain is currently stored in Québec. This combination pattern was also seen in 025, but when it was sequenced, it turned out to be *prn*2_1. This suggests that there may have been an overflow from one of the wells that contained *prn*1_1.

Prn3_1, one of the types originally found in The Netherlands was found in BpeXba013, BpeXba017, BpeXba026, and BpeXba028. Its SSCP pattern is shown in Figure 3.8. The pattern for *prn*3_1 is similar to the pattern for *prn*2_1, in that it has only one band. But the mobility of *prn*3_1 is in between *prn*9_1 and *prn*1_1, while the mobility of *prn*1_1 is the same as the second band of the *prn*9_1 pattern.

Since only one strain from each of the top 30 pulsed-field types was screened we were unsure if it could be assumed that all of the strains in a given pulsed-field type had the same antigenic profile. To determine this, we chose to look at four pulsed-field types more closely; BpeXba001, the most prevalent type isolated in Alberta and Québec combined in the ten-year period; BpeXba002, the most commonly isolated type in Alberta; BpeXba003, the most predominantly isolated type in Québec, and BpeXba008, a

type that was beginning to dominate the strains being isolated in Alberta in 1995. By 1996 it composed 76% of the strains being isolated and then dropped until 1999 to 52.8% (data not shown). Representative surveys of the strains in these four pulsed-field types were tested, in which 25% per year with a maximum of 12, were screened.

Looking at the two antigens separately, pertactin types within BpeXba001 show the most heterogeneity, while BpeXba002 and BpeXba003 pertactin types show limited heterogeneity, and Bpexba008 pertactin types are homogenous (Figure 3.9). The *ptxS1* types of the strains within BpeXba001, 002, 003, 008 are much more uniform than the pertactin types (Figure 3.9).

A summary of the actual number of isolates tested from each of the four pulsed-field types is shown in Table 3.3. If the data are grouped according to whether their *prn/ptxS1* are considered "old", "transitional", or "new", most of the BpeXba001 and BpeXba002 strains sampled are "new" types, 89% and 82% respectively, with a few "transitional", 8% and 9% respectively, and "old" types, both at 3% (Tables 3.4 and 3.5). The BpeXba003 strains looked at are almost entirely "old" types with only one "new" type (Table 3.6), while the BpeXba008 strains that were screened are entirely "new" types (Table 3.7).

The time the pulsed-field type appears in the population may dictate what antigenic profile it will have. This is shown with the BpeXba001, Bpexba002, and BpeXba008 data (Tables 3.4, 3.5, and 3.7). For example BpeXba008 emerged in 1990, therefore all of BpeXba008 strains have the "new" profile. For strains that have been present in the population for some time, like BpeXba001, the year the strain was isolated, will dictate what antigenic type it will have, resulting in a variety of antigenic profiles

within the pulsed-field type. As shown in The Netherlands and the United States (Cassidy, P., 2000 , Mooi, F, 1998), most likely BpeXba001 strains circulating in Canada until the early 1970's all possessed an "old" antigenic profile. BpeXba001 strains being isolated from the mid 1970's until the 1984 probably contained "old" and "transitional" profiles, with "new" profiles emerging in the early 1980's. As shown in Table 3.4, from 1984 to 1990, BpeXba001 strains sampled had mostly "new" profiles with a few "old" and "transitional" ones still around. From 1990 it can be seen that the BpeXba001s with the "new" antigenic profile were solely being isolated. This pattern seen in BpeXba001 can also be seen within BpeXba002 (Table 3.5). These patterns seen with BpeXba001 and Bpexba002 support the hypothesis that the "new" antigenic profiles would give an evolutionary advantage to the strains possessing them, over strains with "transitional" profiles; therefore strains containing the "new" antigenic profiles would eventually become the dominating strains because they are able to evade vaccine-induced immunity better. That is, strains with two "new" antigens are better off than strains with one "old" and one "new" antigen. This data from BpeXba001 and BpeXba002 also suggests that the genomic rearrangement of *B. pertussis*, resulting in different pulsed-field patterns, and the addition or deletion of bases and repeats resulting in different antigenic types, are independent of each other. Correlation between antigenic type and pulsed-field type may not be as clear for types that have been around for greater than 10 years compared to types that have just recently emerged.

BpeXba003, the dominating pulsed-field type in Québec, is different from BpeXba001 and BpeXba002, in that the BpeXba003 strains tested between 1985 and 1994 all have "old" antigenic profiles (Table 3.6). The BpeXba001 and BpeXba002

strains looked at were from Alberta, so the pattern seen with these two types may not be the same pattern seen with them in Québec. These differences between the two provinces may be due to the use of a different vaccine. Armand-Frappier (A-F) manufactured the vaccine used in Québec until 1985. Although the antigenic profile of the Armand Frappier vaccine is unknown we could reason, based on the experience in The Netherlands and the United States that the vaccine strains used contained the "old" antigens *prn*1/*ptxS1*D. The vaccine used in the rest of Canada had two strains, which probably had antigenic profiles of *prn*1/*ptxS1*D and *prn*1/*ptxS1*B. If *prn*1/*ptxS1*D was the only antigenic profile in the A-F vaccine, there may not have been the same type of vaccine-induced pressure in Québec as there was in Alberta and the rest of Canada. That is, there was pressure to cause the switch to *ptxS1*B, but not to *ptxS1*A. Until Québec started using the same vaccine as the rest of Canada, introducing the other *ptxS1* type, there would be no selective pressure against *ptxS1*B.

Even though it seems that pulsed-field type and antigens do not correlate for every pulsed-field type they were still grouped together. Table 3.8 shows the number and percentages of the different pertactin and S1 pertussis toxin types of the top 30 pulsed-field types isolated between 1985 and 1994. Québec has 38% "new" pertactin types versus 62% "old" pertactin types and 56% "new" pertussis toxin types versus 44% "old" pertussis toxin types. Alberta has 54% "new" pertactin types ($prn2_1$, 3_1, 9_1) versus 47% "old" pertactin types ($prn1_1$), and a 69% "new" S1 pertussis toxin type (*ptxS1*A) versus 31% "old" S1 pertussis toxin type (*ptxS1*B). Also, from 1995 to 1999 in Alberta, 84%, 86%, 89.6%, 91.7%, and 84.9% of the pulsed-field types isolated in each respective year had $prn2_1$ and *ptxS1*A (data not shown). The difference between Albert and Québec

once again could be attributed to the use of different vaccines in the two provinces. When Québec and Alberta are combined, there are 46% and 64% "new" pertactin and pertussis toxin types respectively and 53% and 36% "old" pertactin and pertussis toxin types in Canada.

Compared to the data described for Alberta and Québec, the data from the United States are slightly different. As shown by Cassidy *et al.*, in the United States, during the time period of 1935-1974, all 21 (100%) strains had the *prn*1_1 pertactin type, and 37 of 39 (95%) strains between 1975 and 1987 had *prn*1_1 type. *Prn*2_1 was first isolated in 1981, and by 1989-1999, 64 of the 92 (70%) strains typed had the *prn*2_1 type. No *prn*3_1 was found. It was also shown that during the period 1935-1974, three of the twenty-one strains (14%) (all isolated before 1946) had the *ptxSI*D pertussis toxin type, while the other seventeen strains (81%) had *ptxSI*B. *PtxSI*A appeared in 1970, and from 1975-1987 was found in 25 of 39 (64%) strains, and from 1989-1999 it was found in 72 of 92 (78%) strains. All strains, except one, isolated between 1996 and 1999 were *ptxSI*A (98%) (Cassidy, P., 2000).

Trends in The Netherlands differ both from ours, and that of the United States. Mooi *et al.* have shown that from 1949 to 1972, 100% of the isolates had the *prn*1_1 pertactin type. Between 1976-1990, *prn*1_1 was found in 83% of the isolates, *prn*2_1 was found in 11% and *prn*3_1 was in 6% of the isolates. By 1989 to 1996, *prn*1_1 was only found in 13% of isolates, while 36% and 51% of the isolates contained *prn*2_1 and *prn*3_1, respectively (Mooi, F., 1998). Between 1949 and 1954 58% of the strains isolated had *ptxsI*B, and 42% had *ptxSI*D. By the late 1970s, *ptxSI*A appeared, and between 1978 and 1985 was found in 80% of the isolates, while *ptxSI*B was found in only 20% and

*ptxS1*D was no longer found. From 1990-1996, *ptxS1*A comprised 88% of isolates and 12% contained *ptxS1*B (Mooi, F., 1998).

The trends seen in The Netherlands, the United States, and Alberta and Québec are difficult to compare because the time periods looked at differ for each of the three data sets. There is more consistency in the time periods between the United States and The Netherlands, so it is easier to evaluate the trends in these two countries. Because of the limited availability of strains in Alberta and Québec, it has not been possible to determine what the pertactin and S1 pertussis toxin trend was before 1985. The only similarity between the three sets of data is that by 1996-1999, "new" *prn* and "new" *ptxS1* types were the most common types being isolated in all three countries.

The top 30 pulsed-field types from Canada (Alberta and Québec) were assigned antigenic profiles even though they were not absolute correlates (they were based on the dominant types). They were grouped according to the combined total antigenic profiles of *prn/ptxS1*. Table 3.9 shows that 54% of Alberta strains have a "new" antigenic profile, 16% have a "transitional" profile, and 31% have an "old" profile. Also, from 1995-1999, 84%, 86%, 89.6%, 91.7%, and 84.9% of strains isolated in each respective year had the "new" antigenic profile (data not shown). In Québec, 38% of the strains between 1985 and 1994 are "new", 18% are "transitional", and 44% are "old". Alberta and Québec combined have 46% "new" strains, 18% "transitional" and 36% "old". The different distributions of "old" versus "new" strains in Québec once again suggest the evolution of the strains in this province to be quite different from the evolution of strains in Alberta, as previously described.

In comparison, as shown by Cassidy, P., 2000 , *et al.* strains isolated in the United States, before 1993, either had the "transitional" type *prn1/ptxS1*A or the "old" type *prn1/ptxS1*B. There were 50 "old" strains that were isolated from 1946-1994, while the 33 transitional strains were isolated from 1978-1993. After 1993, strains with the "new" antigenic profile were seen almost exclusively (64/65), while only one "new" strain was isolated before 1993, in 1983. The United States and Canada differ in that the combination of two "new" antigens resulting in a "new" antigenic profile really did not appear in the U.S. until 1993. In Canada, strains with the "new" profile were seen since 1985 as shown by the BpeXba001 data (Table 3.4).

There is evidence of vaccine-induced pressure on other organisms. Preliminary evidence regarding measles virus and the measles vaccine show that neutralization ability of sera against 20 measles viruses varied among adolescent vaccinees from Luxembourg, and Nigerian women with naturally acquired immunity (Klingele, M., 2000). Although there are many reasons that can be attributed to this phenomenon, two observations were made suggesting antigenic variation may have been involved. First, all the viruses in the study were neutralized by the sera from the adolescents and women better than the vaccine strains and second, 2 of the measles viruses tested had unique amino acid changes in their hemagglutinin protein when compared to other strains isolated during the same outbreak.

It can be concluded from the data presented that antigenic variation has occurred in Alberta and Québec. Unfortunately, since strains prior to 1985 were unattainable, the time of transition from "old" to "new" antigens is unknown. Based on data from The

Netherlands, Finland, Italy, and the United States, it is likely *ptxS1*A appeared in the late 1970s, in Canada and *prn*2 and *prn*3 in the early 1980s.

Primers	Sequence (5′-3′)	Gene	Position
PF*	GCCAATGTCACGGTCCAA	*prn*	649-666
PR*	CGGATTCAGGCGCAACTC	*prn*	2076-2059
PF2	TGGTGCTGCGCGACACCAAC	*prn*	746-766
PR2	GACTGGGCGAGCTCCAAGCT	*prn*	1073-1053
S1F+	TAGGCACCATCAAAACGCAG	*ptxS1*	474-493
S1R+	TCAATTACCGGAGTTGGGCG	*ptxS1*	1350-1330
S1FM+	ACAATGCCGGCCGTATCCTC	*ptxS1*	949-965
S1BF	ATTCCAACGCTCGCTACGTC	*ptxS1*	1090-1109

Table 3.1. Primers used for amplifying the pertactin gene and the S1 subunit of pertussis toxin gene. Pertactin primers marked with an * are primers AF and BR taken from Mooi *et. al*, 1998. S1 primers marked with a + are taken directly from Mooi *et. al*, 1998.

Table 3.2. The pertactin and S1 pertussis toxin types for the top 30 pulsed-field types in Alberta and Québec circulating between 1985 and 1994. The distribution of the top 30 within the two provinces is also shown.

Strain	Type of Pertactin (*prn*)			Type of pertussis toxin S1 (*ptxS1*)			Total in Alberta	Total in Québec	Total in Alberta and Québec
	1	2	3	A	B	D			
BpeXba001		X		X			315	228	543
BpeXba002		X		X			335	129	464
BpeXba003	X				X		31	304	335
BpeXba004	X			X			84	172	256
BpeXba005	X			X			159	60	219
BpeXba006	X				X		202	3	205
BpeXba007	X			X			125	26	151
BpeXba008		X		X			56	71	127
BpeXba009	X				X		94	22	116
BpeXba010	X				X		37	57	94
BpeXba011		X		X			96	0	96
BpeXba012	X			X			58	21	79
BpeXba013			X	X			32	47	79
BpeXba014	X				X		8	68	76
BpeXba015	X				X		43	14	57
BpeXba016	X				X		0	48	48
BpeXbA017			X	X			45	2	47
BpeXba018		X		X			38	1	39
BpeXba019	X				X		30	5	35
BpeXba020		X		X			25	7	32
BpeXba021		X		X			2	30	32
BpeXba022	X				X		0	33	33
BpeXba023	*prn*9			X			14	9	23
BpeXba024	X				X		21	2	23
BpeXba025		X		X			0	21	21
BpeXba026			X	X			19	2	21
BpeXba027	X				X		2	26	28
BpeXba028			X	X			13	6	19
BpeXba029	*prn*9			X			17	1	18
BpeXba030	*prn*1+2			X			0	18	18
							1901	1433	3334
Total of all strains isolated in the 10 year period							2022	1575	3597

Alberta BpeXba001		Alberta BpeXba002		Québec BpeXba003		Alberta BpeXba008	
Strains Tested	Total/Year	Strains Tested	Total/Year	Strains Tested	Total/Year	Strains Tested	Total/Year
3	3	0	0	3	8	0	0
7	27	2	2	4	11	0	0
2	3	0	0	3	9	0	0
3	3	4	4	6	21	0	0
7	33	9	34	7	22	0	0
10	112	10	69	7	28	1	1
3	10	10	49	10	51	0	0
10	46	10	44	10	44	0	0
10	50	10	80	10	80	5	5
7	28	10	53	8	30	12	50
62	315	65	335	68	304	18	56

Table 3.3. Summary of the actual number of isolates tested and actual the total/year for each of the four pulsed-field types, BpeXba001, Bpexba002, BpeXba003, and BpeXab008.

	Alberta BpeXba001 Pertactin/Pertussis Toxin S1 Types							
Year	***prn1₁/ptxS1D*** "old"	***prn1₁/ptxS1B*** "old"	***prn1₁/ptxS1A*** "transitional"	***prn2₁/ptxS1A*** "new"	***prn3₁/ptxS1A*** "new"	***prn9₁/ptxS1A*** "new"	**Strains Tested**	**Total/Year**
1985	0	0	0	3	0	0	3	3
1986	0	0	0	7	0	0	7	27
1987	0	0	0	2	0	0	2	3
1988	0	1	1	0	0	1	3	3
1989	0	0	2	5	0	0	7	33
1990	0	1	2	5	0	2	10	112
1991	0	0	0	2	0	1	3	10
1992	0	0	0	7	0	3	10	46
1993	0	0	0	9	1	0	10	50
1994	0	0	0	7	0	0	7	28
Totals	0	2	5	47	1	6	62	315

Table 3.4. Shows the Alberta BpeXba001 strains grouped according to their antigenic profiles. Most of the strains looked at have "new" antigens (89%), a few are "transitional" (8%), and 3% are "old" types.

	Alberta BpeXba002 Pertactin/Pertussis Toxin S1 Types							
Year	***prn1₁/ptxS1D*** **"old"**	***prn1₁/ptxS1B*** **"old"**	***prn1₁/ptxS1A*** **"transitional"**	***prn2₁/ptxS1A*** **"new"**	***prn3₁/ptxS1A*** **"new"**	***prn9₁/ptxS1A*** **"new"**	**Strains Tested**	**Total/Year**
1985	0	0	0	0	0	0	0	0
1986	0	1	0	1	0	0	2	2
1987	0	0	0	0	0	0	0	0
1988	0	1	1	2	0	0	4	4
1989	0	0	5	4	0	0	9	34
1990	0	0	0	8	0	1	10	69
1991	0	0	0	10	0	0	10	49
1992	0	0	0	10	0	0	10	44
1993	0	0	0	10	0	0	10	80
1994	0	0	0	7	0	0	10	53
Totals	0	2	6	52	0	1	65	335

Table 3.5. Shows the antigenic profiles of the Alberta BpeXba002 strains screened. BpeXba002 follows a similar pattern to Bpexba001, with 82% "new" types, 9% "transitional" and 3% "old" types.

	Québec BpeXba003 Pertactin/Pertussis Toxin S1 Types							
Year	***prn1₁/ptxS1D*** "old"	***prn1₁/ptxS1B*** "old"	***prn1₁/ptxS1A*** "transitional"	***prn2₁/ptxS1A*** "new"	***prn3₁/ptxS1A*** "new"	***prn9₁/ptxS1A*** "new"	**Strains Tested**	**Total/Year**
1985	0	3	0	0	0	0	3	8
1986	0	4	0	0	0	0	4	11
1987	0	3	0	0	0	0	3	9
1988	0	6	0	0	0	0	6	21
1989	0	7	0	0	0	0	7	22
1990	0	7	0	0	0	0	7	28
1991	0	10	0	0	0	0	10	51
1992	0	9	0	0	1	0	10	44
1993	0	10	0	0	0	0	10	80
1994	0	8	0	0	0	0	8	30
Totals	0	67	0	0	1	0	68	304

Table 3.6. Shows the antigenic profiles of the Québec BpeXba003 strains screened. 67/68 all of the strains looked at had an "old" profile, only one strain showed a "new" profile.

	Alberta BpeXba008 Pertactin/Pertussis Toxin S1 Types							
Year	***prn1₁/ptxS1D*** "old"	***prn1₁/ptxS1B*** "old"	***prn1₁/ptxS1A*** "transitional"	***prn2₁/ptxS1A*** "new"	***prn3₁/ptxS1A*** "new"	***prn9₁/ptxS1A*** "new"	**Strains Tested**	**Total/Year**
1985	0	0	0	0	0	0	0	0
1986	0	0	0	0	0	0	0	0
1987	0	0	0	0	0	0	0	0
1988	0	0	0	0	0	0	0	0
1989	0	0	0	0	0	0	0	0
1990	0	0	0	1	0	0	1	1
1991	0	0	0	0	0	0	0	0
1992	0	0	0	0	0	0	0	0
1993	0	0	0	5	0	0	5	5
1994	0	0	0	12	0	0	12	50
Totals	0	0	0	18	0	0	18	56

Table 3.7. Shows the antigenic profile of the Alberta BpeXba008 strains screened. All of the strains looked at were "new" antigenic types. BpeXba008 is a relatively new pulsed-field type appearing in the early 1990s that starts to dominate the type of strains being isolated in Alberta beginning in 1995.

	Pertactin Type				S1 subunit of Pertussis Toxin		
	prn1_1	*prn*2_1	*prn*3_1	*prn*9_1	*ptxS1A*	*ptxS1B*	*ptxS1D*
Alberta	894 (47)	867 (46)	109 (6)	31 (2)	1308 (69)	593 (31)	0 (0)
Québec	861 (62)	466 (33)	57 (4)	10 (1)	786 (56)	608 (44)	0 (0)
Combined	1755 (53)	1330 (40)	166 (5)	41 (1)	2094 (64)	1201 (36)	0 (0)

Table 3.8. Total numbers and (percentages) of *prn* and *ptxS1* types within the top 30 pulsed-field types from 1985-1994 in Alberta and Québec.

	*prn*1$_1$/*ptxS1*B "old"	*prn1*$_y$/*ptxS1*A "transitional"	*prn2*$_y$/*ptxS1*A "new"	*prn3*$_y$/*ptxS1*A "new"	*prn9*$_y$/*ptxS1*A "new"	Totals
Alberta	593 (31)	301 (16)	867 (46)	109 (6)	31 (2)	1901
Québec	608 (44)	253 (18)	466 (33)	57 (4)	10 (1)	1394
Combined	1201 (36)	554 (17)	1333 (40)	166 (5)	41 (1)	3295

Table 3.9. Total numbers and (percentages) for each combination of *prn* and *ptxS1* antigenic types within the top 30 pulsed-field types in Alberta and Québec between 1985-1994.

Figure 3.1. Pertactin Region 1 sequences, starting at base 778, for BpeXba001, BpeXba003, BpeXba008, BpeXba011, CCL-1, CCL-2, CCL-3, and CCL-4 show that there are antigenic variants present in strains in Alberta and Québec. These eight strains were then used to establish the protocol for SSCP and the patterns for the pertactin variants.

BpeXba001

778

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC
R G D A P A G G A V P G G A V P G G F G P G G F G P G G F G P G G F G P V

BpeXba003

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGT GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC CTC
R G D A P A G G A V P G G A V P G G A V P G G F G P G G F G P V L

BpeXba008

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC
R G D A P A G G A V P G G A V P G G F G P G G F G P G G F G P G G F G P V

BpeXba011

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC
R G D A P A G G A V P G G A V P G G F G P G G F G P G G F G P G G F G P V

CCL-1

CGC GGG GAC GCG CCT GCC GGC GGT GGG GTT CCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC GTC
R G D A P A G G G V P G G A V P G G A V P G G F G P G G F G P V L

CCL-2

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGT GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC CTC
R G D A P A G G A V P G G A V P G G A V P G G F G P G G F G P V L

CCL-3

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGT GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC CTC
R G D A P A G G A V P G G A V P G G A V P G G F G P G G F G P V L

CCL-4

CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGT GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC CTC
R G D A P A G G A V P G G A V P G G A V P G G F G P G G F G P V L

BpeXba001

193 676

AAT GTG CTC GAC CAT CTG // GTG CGC ATA GCG CCG GTG ATA GGC
N V L D H L V R I A P V I G

BpeXba003

AAT GTG CTC GAC CAT CTG // GTG CGC ATG GCG CCG GTG ATA GGC
N V L D H L V R M A P V I G

BpeXba008

AAT GTG CTC GAC CAT CTG // GTG CGC ATA GCG CCG GTG ATA GGC
N V L D H L V R I A P V I G

BpeXba011

AAT GTG CTC GAC CAT CTG // GTG CGC ATA GCG CCG GTG ATA GGC
N V L D H L V R I A P V I G

CCL-1

AAT GTG CTC GAA CAT CTG // GTG CGC ATG GCG CCG GTG ATG GGC
N V L E H L V R M A P V M G

CCL-2

AAT GTG CTC GAA CAT CTG // GTG CGC ATG GCG CCG GTG GTG GGC
N V L E H L V R M A P V V G

CCL-3

AAT GTG CTC GAC CAT CTG // GTG CGC ATG GCG CCG GTG ATA GGC
N V L D H L V R M A P V I G

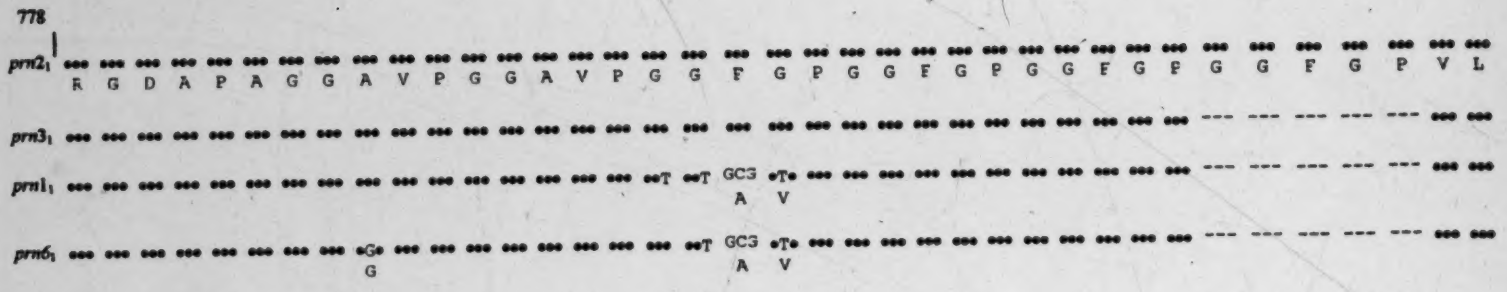
CCL-4

AAT GTG CTC GAA CAT CTG // GTG CGC ATG GCG CCG GTG GTG GGC
N V L E H L V R M A P V V G

Figure 3.2. Two regions in the S1 subunit of pertussis toxin were sequenced for eight strains Bpexba001, BpeXba003, BpeXba008, BpeXba011, CCL-1, CCL-2, CCL-3, and CCL-4 and S1 pertussis toxin variants were found. these strains were used to establish the patterns for the S1 pertussis toxin variants.

Figure 3.3. The pertactin and S1 subunit of pertussis toxin variants found in The Netherlands by Mooi *et al.*, 1998. The sequences of these variants were found to match the sequences of the eight Alberta and Québec strains sequenced, therefore confirming that these variants are also present in Canada. The sequences for *prn6₁* and *ptxS1E* are unique, found only in 18323 the mouse challenge strain and in our CCL-1 strains.

A. Variants of Pertactin (*prn*)


778
prn2₁ ••• ••• •••

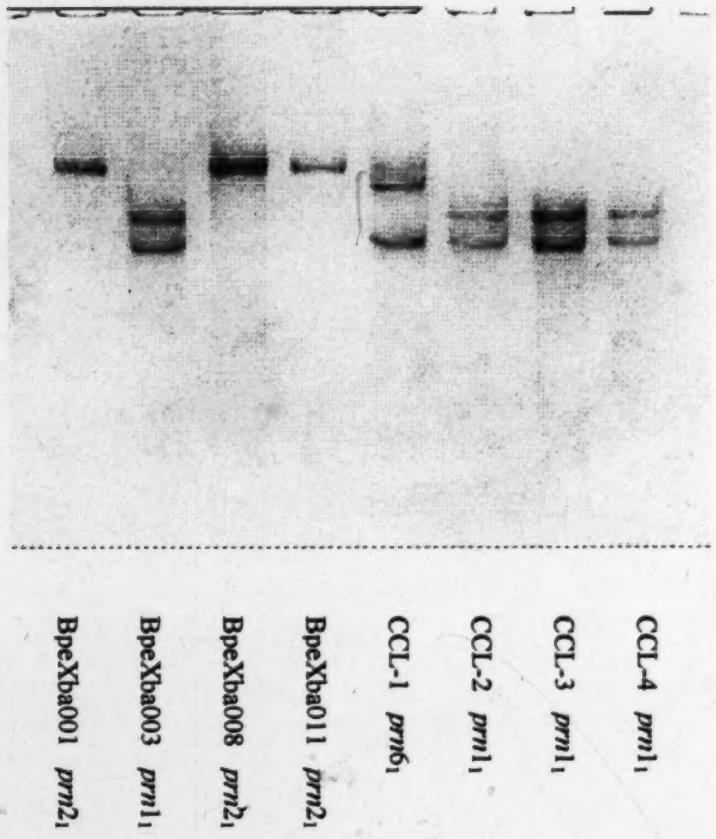


Figure 3.4. The variant pertactin types with their corresponding SSCP patterns for the strains used to establish the SSCP protocol.

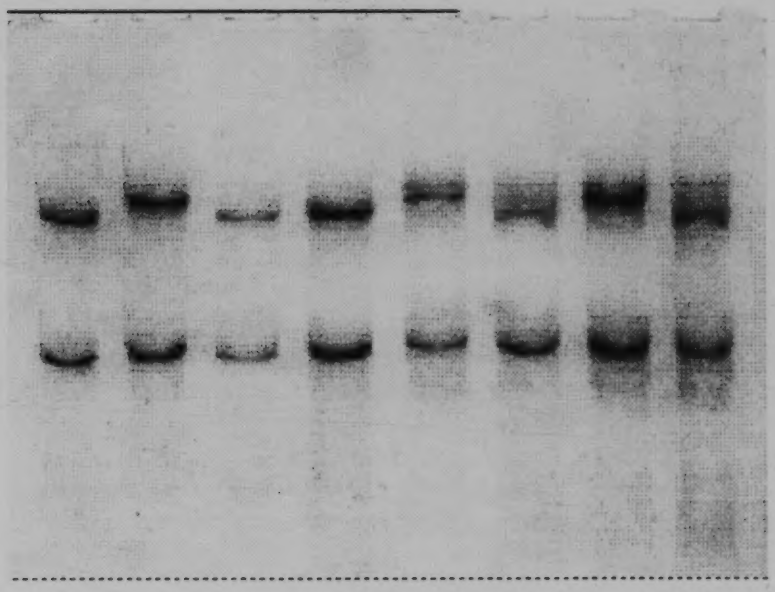


Figure 3.5. The S1 subunit of pertussis toxin variants with their corresponding SSCP patterns from the strains used to establish the SSCP protocol.

Figure 3.6. The SSCP pattern for the new pertactin type *prn*9$_1$ is shown. It is found in strains BpeXba023 and BpeXba029. The pertactin type for strain BpeXba030 showed a mixture of *prn*1 and *prn*2 and the sequencing data was inconclusive. Its pertactin type is yet to be elucidated.

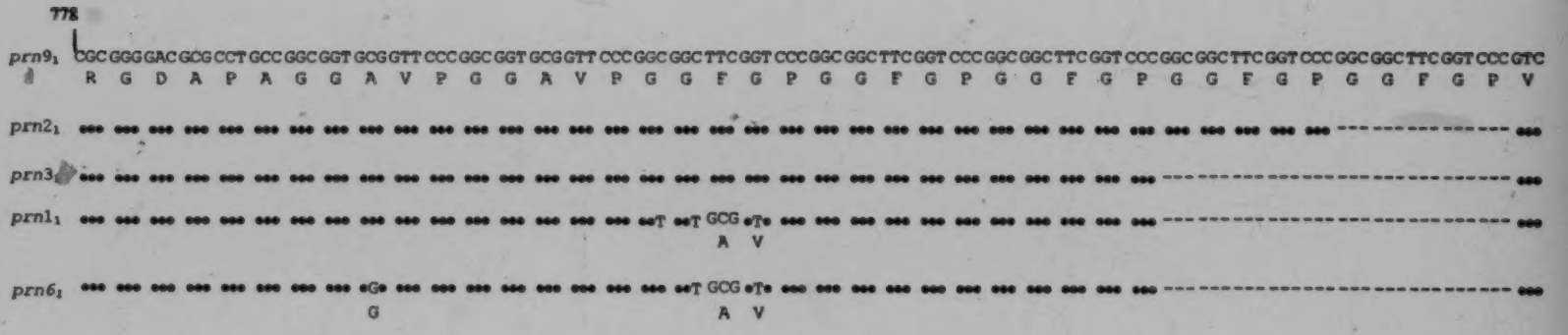
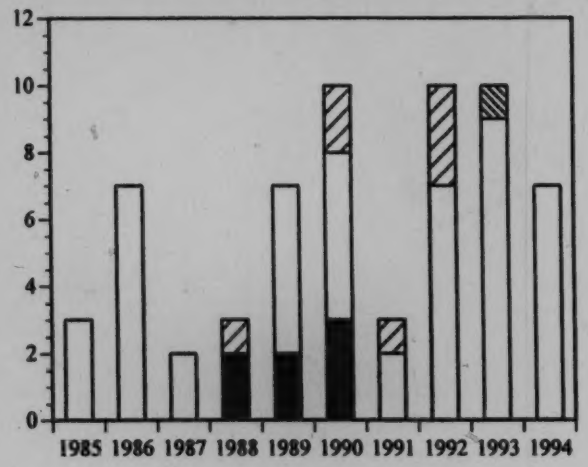

778
prn9₁ CGC GGG GAC GCG CCT GCC GGC GGT GCG GTT CCC GGC GGT GCG GTT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GGC GGC TTC GGT CCC GTC
R G D A P A G G A V P G G A V P G G F G P G G F G P G G F G P G G F G P V
prn2₁
prn3
prn1₁
prn6₁

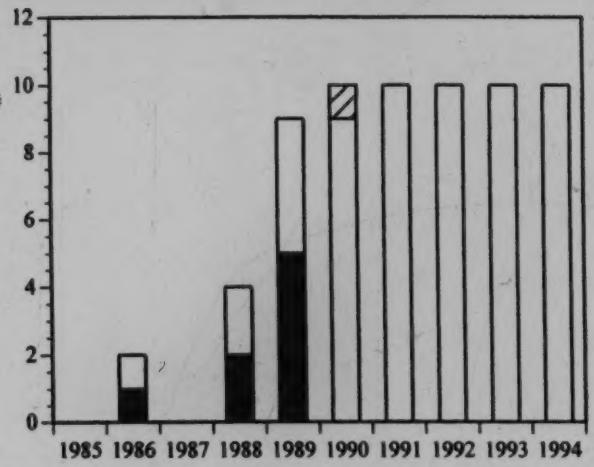
Figure 3.7. The new pertactin type, *prn*9$_1$ found in Alberta and Québec. Its sequence is compared to the sequences of the other pertactin variants, showing that *prn*9$_1$ has an extra GGFGP repeat.

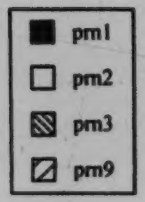
Figure 3.8. The SSCP pattern for pertactin type *prn*3$_1$ is shown. It is found in BpeXba013, BpeXba017, BpeXba026, and BpeXba028.

A. *prn* types by year for **BpeXba001** (n=62)

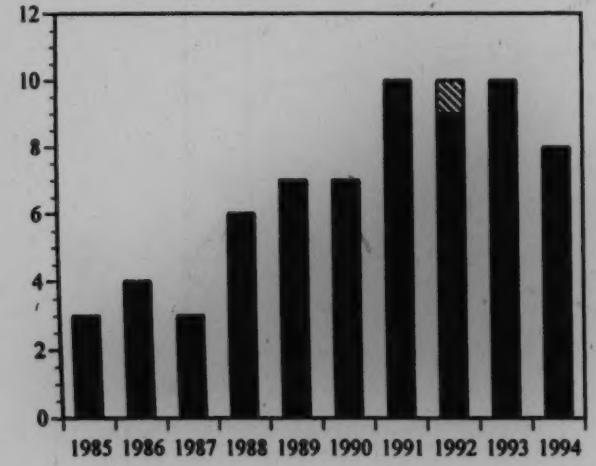


B. *prn* types by year for **BpeXba002** (n=65)

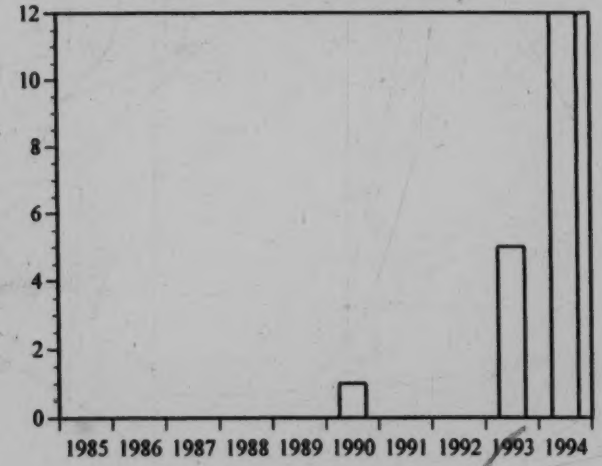




C. *prn* types by year for **BpeXba003** (n=68)



D. *prn* types by year for **BpeXba008** (n=18)



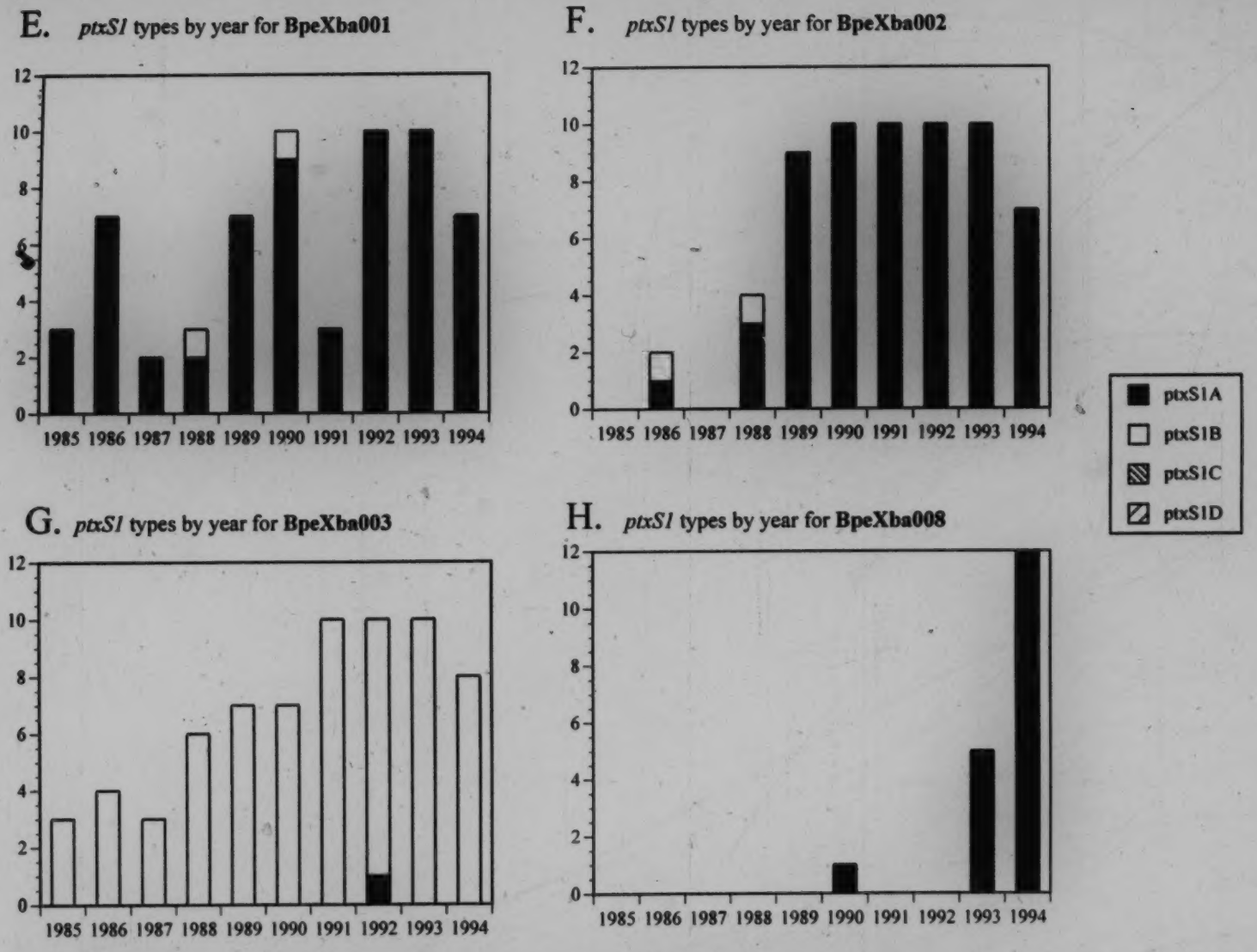


Figure 3.9 Distribution of pertactin (*prn*) types (**A-D**) and S1 pertussis toxin (*ptxS1*)types (**E-H**) within four of the most common PFGE types isolated in Alberta and Québec between 1985-1994. Years in which there were $1 \leq 5$ isolates, all were tested. For years with > 5 isolates, 25% of non-epidemiologically-linked isolates were tested, to a maximum of 12.

CHAPTER IV

SSCP ANALYSIS OF PERTACTIN VARIANTS WITH CAPILLARY ELECTROPHORESIS

Introduction

Conventional SSCP analysis proved to be a good tool to screen *B. pertussis* strains for antigenic variation. One drawback, however, is that it can be time consuming and is limited by the small number of samples that can be run at one time. To speed up the analysis time and the number of samples that can be run, we created a protocol for SSCP analysis with capillary electrophoresis (CE). SSCP analysis with CE can be done using an automated system that uses a fluorescent dye detection system. The automated system (ABI Prism® 310 Genetic Analyzer) can consist of a 48-well tray or a 96-well tray, but is expensive, approximately $500 000. This technique has been used in human genetics to identify p53 point mutations (Atha, D. H., 1998, Katsuragi, K., 1996), and in identification of bacteria species by analyzing 16S rRNA (Ghonzzi, R., 1999, Vaneechoutte, M., 1998). Our goal was to demonstrate proof of concept for using SSCP analysis with CE for rapid determination of *B. pertussis* antigen alleles.

We focused on the pertactin types and aimed for a run time of about thirty minutes, which would increase the number of samples that could be processed in a day for ongoing epidemiological studies. We used a prototype five-capillary machine that was originally designed for sequencing and the five pertactin types; 1_1, 2_1, 3_1, 6_1, and 9_1

to do this. The five-capillary machine was in Dr. Norm Dovichi's lab in the Department of Chemistry at the University of Alberta, and Dr. Woei Tan helped with the scientific background of the technique and with use of the machine. There are also 1-capillary instruments available in Dr. Norm Dovichi's lab. The capillary instruments in Dr. Dovichi's lab were built by his students. The five capillary instrument that I used was built by Dr. JianZhong Zhang. We preferred these studies in Dr. Dovichi's lab with the hope that the parameters we established there would be transferable to a commercial instrument like the one in Medical Genetics.

Results and Discussion

For SSCP analysis using CE, the pertactin genes of BpeXba001, BpeXba003, BpeXba013, BpeXba023, and CCL-1 were amplified using primers PF2 and PR2, both labeled with the fluorescent dye TAMRA. The resulting PCR products are the same 347 bp amplicons used for gel SSCP analysis but have a fluorescent label. At the CE instrument, the five capillaries are filled with a sieving matrix, in this case a commercial polymer called GeneScan. The matrix is the medium needed for the sample to travel through to get to the detector, providing a similar function as agarose when detecting DNA by conventional electrophoresis. When the single-stranded DNA passes the laser, the detector senses the fluorescent signal, which is transformed into peaks on the graph/readout. Three peaks appear on the graph, the first one being the internal standard, and the other two being the two single strands of the denatured DNA.

Prn1_1 was used to establish the parameters that would be used to compare the rest of the pertactin types. The GeneScan polymer was chosen because it is the polymer used

in the automated ABI PRISM® Genetic Analyzer for SSCP analysis by CE (SSCP Analysis: Chapter 7). During the time it took for the GeneScan polymer to be received, Dr. Tan made a cellulose polymer that we tried at concentrations of 3%, 2%, and 1.5%. This cellulose polymer did not give the resolution that we needed, that is, the line did not return to base line between the peaks and the peaks were too close together. The GeneScan polymer has a concentration of 7%. For our use, we diluted it by a 1:1 ratio with the running buffer (1X TBE containing 10% glycerol), resulting in a concentration of 3.5%.

The injection voltage is the voltage at which the sample is introduced electrokinetically into the capillaries. The time of injection can be varied, but 40 seconds was found to be optimal based on previous trials. The injection time dictates how much of the sample is injected into the capillary, longer injection times means more sample in the capillary and a stronger signal. But, too much sample can also result in a broad peak. The injection voltage can also be varied, but for our purposes, 100 V/cm was chosen early and remained.

Capillary length and CE voltage are two other parameters that can be varied. Longer capillaries result in longer analysis time, but can also result in better resolution. Capillary lengths of 60 cm to 45 cm were tested. 45 cm was chosen because it gave a good run time of about 30 minutes and the resolution of the peaks was acceptable. The CE voltage generates the current that carries the sample through the capillaries to the detector end. The optimal CE voltage depends on the length of the capillaries. A high CE voltage like 300 V/cm can be used with longer capillaries of 60 cm. The same CE voltage applied to 50 or 45 cm capillaries results in an increased current, which will

decrease the run time. However, higher current may occasionally result in bubble formation within the capillary and disrupt separation. This can be resolved by decreasing the temperature of the capillaries during the run. We tried the CE voltage at 300 V/cm, 250 V/cm, and 200 V/cm with varying lengths of capillaries.

After trial runs testing the different parameters, the following conditions were chosen: GeneScan commercial polymer at a concentration of 3.5%; CE voltage at 200 V/cm; injection voltage at 100V/cm; injection time at 40 sec; 45 cm capillaries (OD 150 μm, ID 46 μm); 169 bp internal standard (169 IS). These parameters were set up, and four to six runs with the same sample in each of the five capillaries was done for each pertactin type. Two runs with different pertactin types in the five capillaries were also done. The resulting graphs were printed off and analyzed.

The best way to determine if the different prn types can be distinguished from each other is to use the time variable on the horizontal axis of the graph. This is the time that each of the DNA peaks pass out of the capillary by the laser and are detected. The results for capillary 1 are shown in Table 4.1. Table 4.2 shows the results for capillary 2. Table 4.3 and 4.4 are the results for capillaries 4 and 5 respectively. Capillary number three was plugged and not working properly, so there is no data for it.

The tables show all of the runs for each of the different pertactin types grouped by capillary and number of runs per day (between 3-6 runs per day) because there is variability between the five capillaries even when they contain the same antigen. Also, there is inconsistency within a capillary for any of the antigens. The migration times of the different pertactin types are very close, and because of the variability within a capillary, there is considerable overlap. Even comparing the relative mobility of the

sample peaks to the internal standard is inconsistent for a given pertactin type and capillary. This makes pairing a specific migration time with a specific antigenic type impossible. A possible remedy would be to use longer capillaries. Capillary length influences migration time and separation, so longer capillaries would mean better separation but they would also mean an increase in run time. Unfortunately, our time with the CE machine was limited to two months so there was no time to troubleshoot the parameters. Therefore, our attempt at devising a protocol for SSCP analysis with CE was unsuccessful, due to the lack of sufficient resolution.

In large-scale epidemiologic studies that include analysis of substantial numbers of strains, the purchase of an automated system like the ABI PRISM 310 Genetic Analyzer would be cost effective and useful because it would decrease analysis time. The system uses a 48 or 96 well tray and includes software to analyze the data, allowing alignment of the internal standard controls in each capillary, hence the sample peaks, which eliminates lane-to-lane variability.

If the SSCP analysis with CE on the automated system is not possible, another option would be to do fluorescent SSCP analysis on a automated DNA sequencer like the ABI PRISM 377 DNA sequencer. This technique is similar to slab gel SSCP in that samples are run on acrylamide gels, but on a larger scale. The difference is that the samples are fluorescently labeled, similar to CE. The endpoint is a graph/readout with colored peaks corresponding to the different lanes. Some advantages of using this machine are: 1) similar to other automated machines, an internal control can be used to align the data to eliminate lane-to-lane variability. 2) The two DNA strands can be labeled with a different color. This results in data being easier to interpret, and residual

double stranded DNA can be distinguished from single stranded. 3) If different mutations are being looked for on the same DNA, then samples can be pooled. The primers from each region can be labeled with a different color, for example, the pertactin primers can be labeled with TAMRA, and the S1 primers can be labeled with FAM. The pertactin and S1 PCR products from one strain can then be pooled into one lane.

Run No.	*Prn* Type	Time of IS (min)	Time of peak A (min)	Time of peak B (min)	Time btwn IS and A (min)	Time btwn IS and B (min)	Time btwn A and B (min)
1	1_1	27.50	30.60	32.80	3.10	5.30	2.20
2	1_1	27.60	30.75	33.10	3.15	5.50	2.35
3	1_1	27.40	30.60	32.90	3.20	5.50	2.30
4	1_1	30.00	33.50	36.00	3.50	6.00	2.50
5	1_1	29.80	33.30	35.80	3.50	6.00	2.50
6	1_1	29.90	33.55	36.05	3.65	6.15	2.50
1	2_1	27.80	30.90	33.60	3.10	5.80	2.70
2	2_1	27.90	30.80	33.80	2.90	5.90	3.00
3	2_1	28.20	31.00	33.70	2.80	5.50	2.70
4	2_1	27.70	30.55	33.30	2.85	5.60	2.75
5	2_1	29.50	32.90	36.30	3.40	6.80	3.40
1	3_1	30.10	33.10	36.85	3.00	6.75	3.75
2	3_1	28.30	31.30	33.85	3.00	5.55	2.55
3	3_1	28.50	31.30	34.00	2.80	5.50	2.70
4	3_1	28.75	31.60	34.50	2.85	5.75	2.90
1	9_1	28.65	31.80	34.80	3.15	6.15	3.00
2	9_1	28.55	31.70	34.80	3.15	6.25	3.10
3	9_1	28.65	31.80	34.80	3.15	6.15	3.00
1	6_1	--	31.20	34.20	--	--	3.00
2	6_1	28.40	31.20	34.00	2.80	5.60	2.80
3	6_1	29.70	32.00	34.90	2.30	5.20	2.90
4	6_1	29.20	32.05	35.00	2.85	5.80	2.95

Table 4.1. Results for capillary 1. Capillary 1 from every run completed was grouped together and then grouped within the table according to pertactin type. It can be seen that for a certain pertactin type, there is a lot of variability within the same capillary with regards to time that the peak is detected. If the different pertactin types are compared within capillary 1, it can be seen that their times of detection are too close together to be able to differentiate each of the pertactin types.

Run No.	*Prn* Type	Time of IS (min)	Time of peak A (min)	Time of peak B (min)	Time btwn IS and A (min)	Time btwn IS and B (min)	Time btwn A and B (min)
1	1_1	23.70	25.80	27.55	2.10	3.85	1.75
2	1_1	24.10	25.80	28.10	1.70	4.00	2.30
3	1_1	27.00	30.20	32.30	3.20	5.30	2.10
4	1_1	28.50	32.00	34.40	3.50	5.90	2.40
5	1_1	27.10	30.20	32.40	3.10	5.30	2.20
1	2_1	26.95	28.90	31.55	1.95	4.60	2.65
2	2_1	26.70	29.50	32.30	2.80	5.60	2.80
3	2_1	25.30	27.90	30.40	2.60	5.10	2.50
4	2_1	26.80	29.60	32.20	2.80	5.40	2.60
5	2_1	27.80	31.00	34.00	3.20	6.20	3.00
1	3_1	27.40	30.00	32.40	2.60	5.00	2.40
2	3_1	26.10	29.00	31.20	2.90	5.10	2.20
3	3_1	26.00	28.50	31.10	2.50	5.10	2.60
4	3_1	25.80	28.20	30.75	2.40	4.95	2.55
1	9_1	26.30	29.30	32.10	3.00	5.80	2.80
2	9_1	26.10	28.90	31.60	2.80	5.50	2.70
3	9_1	26.20	29.10	31.90	2.90	5.70	2.80
1	6_1	--	28.70	31.40	--	--	2.70
2	6_1	26.10	28.60	31.30	2.50	5.20	2.70
3	6_1	26.65	29.75	32.20	3.10	5.55	2.45
4	6_1	26.80	29.40	32.20	2.60	5.40	2.80

Table 4.2. Results for capillary 2. Capillary 2 from every run completed was grouped together and then grouped within the table according to pertactin type. It can be seen that for a certain pertactin type, there is a lot of variability within capillary 2 with regards to time that the peak is detected. If the different pertactin types are compared, it can be seen that their times of detection are too close together to be able to differentiate each of the pertactin types.

Run No.	*Prn* type	Time of IS (min)	Time of peak A (min)	Time of peak B (min)	Time btwn IS and A (min)	Time btwn IS and B (min)	Time btwn A and B (min)
1	1_1	26.60	29.55	32.10	2.95	5.50	2.55
2	1_1	26.50	29.80	32.00	3.30	5.50	2.20
3	1_1	29.30	32.80	35.40	3.50	6.10	2.60
4	1_1	26.60	29.70	32.10	3.10	5.50	2.40
1	2_1	26.75	30.0	32.80	3.25	6.05	2.80
2	2_1	27.30	30.20	33.20	2.90	5.90	3.00
3	2_1	25.40	27.80	30.20	2.40	4.80	2.40
4	2_1	26.80	29.80	32.80	3.00	6.00	3.00
5	2_1	28.40	31.80	35.10	3.40	6.70	3.30
6	2_1	27.60	30.80	33.45	3.20	5.85	2.65
1	3_1	27.70	30.50	34.10	2.80	6.40	3.60
2	3_1	26.40	29.50	31.70	3.10	5.30	2.20
3	3_1	26.00	28.80	31.30	2.80	5.30	2.50
4	3_1	26.25	28.90	31.10	2.65	4.85	2.20
5	3_1	27.20	30.50	32.85	3.30	5.65	2.35
1	9_1	26.60	29.70	32.60	3.10	6.00	2.90
2	9_1	26.70	29.70	32.65	3.00	5.95	2.95
3	9_1	26.35	29.85	32.30	3.50	5.95	2.45
1	6_1	--	29.00	31.90	--	--	2.90
2	6_1	26.40	29.00	31.90	2.60	5.50	2.90
3	6_1	27.50	30.30	33.30	2.80	5.80	3.00
4	6_1	26.90	29.50	32.40	2.60	5.50	2.90

Table 4.3. Results for capillary 4. Capillary 4 from every run completed was grouped together and then grouped within the table according to pertactin type. It can be seen that for a certain pertactin type, there is a lot of variability within the capillary with regards to time that the peak is detected. If the different pertactin types are compared, it can be seen that their times of detection are too close together to be able to differentiate each of the pertactin types.

Run No.	*Prn* type	Time of IS (min)	Time of peak A (min)	Time of peak B (min)	Time btwn IS and A (min)	Time btwn IS and B (min)	Time btwn A and B (min)
1	1_1	25.60	28.20	30.35	2.60	4.75	2.15
2	1_1	26.10	29.30	31.50	3.20	5.40	2.20
3	1_1	27.90	31.10	33.50	3.20	5.60	2.40
4	1_1	27.00	30.20	32.40	3.20	5.40	2.20
1	2_1	26.40	29.50	32.15	3.10	5.75	2.65
2	2_1	26.40	29.30	31.90	2.90	5.50	2.60
3	2_1	26.20	29.00	31.70	2.80	5.50	2.70
4	2_1	26.20	29.10	31.90	2.90	5.70	2.80
5	2_1	29.90	33.30	36.70	3.40	6.80	3.40
1	3_1	28.30	31.20	34.70	2.90	6.40	3.50
2	3_1	27.20	30.35	32.60	3.15	5.40	2.25
3	3_1	27.10	29.95	32.50	2.85	5.40	2.55
4	3_1	27.00	29.60	32.30	2.60	5.30	2.70
5	3_1	28.00	31.40	33.80	3.40	5.80	2.40
1	9_1	27.70	30.80	33.85	3.10	6.15	3.05
2	9_1	27.70	30.90	33.90	3.20	6.20	3.00
3	9_1	27.50	30.60	33.60	3.10	6.10	3.00
1	6_1	--	30.10	33.00	--	--	2.90
2	6_1	27.40	30.20	32.90	2.80	5.50	2.70
3	6_1	28.10	30.95	34.00	2.85	5.90	3.05
4	6_1	28.45	31.40	34.50	2.95	6.05	3.10

Table 4.4. Results for capillary 5. Capillary 5 from every run completed was grouped together and then grouped within the table according to pertactin type. It can be seen that for a certain pertactin type, there is a lot of variability within the same capillary with regards to time that the peak is detected. If the different pertactin types are compared, it can be seen that their times of detection are too close together to be able to differentiate each of the pertactin types.

CHAPTER V

ANTIGENIC VARIATION IN ADENYLATE CYCLASE TOXIN AND MAJOR PORIN

Introduction

Bordetella pertussis has many virulence factors that are thought to contribute to the pathogenesis of whooping cough. All of these antigens and others not specifically associated with virulence were included in the original whole-cell vaccines. Because of this, we were interested to see if any other dominant antigens other than pertactin and the S1 subunit of pertussis toxin, were affected by vaccine-induced evolution. Two antigens were chosen, adenylate cyclase toxin (CyaA) and major porin (Omp40). Adenylate cyclase was selected because it is known to be an important virulence factor for *B. pertussis*. Without it, *B. pertussis* is avirulent in mice (Weiss, A., 1983). Anti-CyaA antibodies are found in humans after immunization and infection suggesting that it may be important in immunity against whooping cough (Arcinega, J., 1991, Farfel, Z., 1991, Thomas, M., 1989). CyaA is not part of any ACV and there is a question as to whether it should be included.

Omp40 is a trimeric protein that forms pores in the membrane of *B. pertussis* and is found in pathogenic and nonpathogenic strains (Li, Z. M., 1991), i.e.; Omp40 is not regulated by the Bordetella virulence gene locus (*bvg*). Antibodies against Omp40 were found in human sera after immunization and disease, but the significance of this is unknown (Anwar, H., 1991). It also is not included in the new ACVs. We were

interested to see if antigenic variation might occur in an antigen that is not regulated by *bvg* and has no known role in virulence

Based on our success with pertactin and the S1 subunit of pertussis toxin, we decided to use SSCP as a screening method to determine if there was any antigenic variation in adenylate cyclase or major porin. The two genes were screened in seven strains, BpeXba001, BpeXba003, BpeXba008, CCL-1, CCL-2, CCL-3, and CCL-4. As determined earlier, these seven strains represent a sample of the most divergent "old" and "new" types of isolates and would be most likely to show differences in Omp40 and CyaA.

Results and Discussion

The sequence of Omp40 is known. It is a small gene that encodes a 365 amino-acid polypeptide. The coding region of major porin is about 1156 bp, so the entire region was screened. Primers were generated along the coding region in a step-wise fashion with products ranging between 250-300 bases (Figure 5.1). The primers were made so that the reverse primer of pair one overlapped with the forward primer of pair two and so forth. The primers used are shown in Table 5.1.

DNA from each of the seven *B. pertussis* strains was amplified using the 5 sets of porin primers using the same protocol that was used for amplifying the pertactin gene. To look for sequence variation, the amplicons were separated using the SSCP protocol established for pertactin and pertussis toxin. The seven strains were run on the same SSCP gel so the amplicons from each primer set could be directly compared. If a unique pattern was seen in any of the strains, it would be sequenced. Figure 5.2 shows the

resulting SSCP patterns for primer pairs POR 1F 1R (A), POR 2F 2R (B), POR 3F 3R (C), and POR 4F 4R (D). Primers POR 1F 1R include bases 406 to 692. The start codon for the porin gene is at position 448. Primers POR 2F 2R include bases 673 to 971. There were no sequence variations found within these two regions. POR 3F 3R include bases 949 to 1235 and POR 4F 4R include bases 1216 to 1491 of the Omp40 gene. No sequence variations were found in these regions. The last set of primers, POR 5F POR 5R include bases 1355 to 1636. The stop codon for the porin gene is at position 1604. For this region the resulting SSCP patterns for the seven strains are shown in Figure 5.3. No sequence variation was found in this area either. However, each region showed a unique profile, based on the sequence of that specific region.

The absence of sequence variation in Omp40, especially in strains CCL-2, CCL-3, and CCL-4 that are considered "old" according to their *prn* and *ptxS1* profiles, was expected since Omp40 is probably not a major virulence factor of *B. pertussis*. If the hypothesis of vaccine-induced selective pressure is true, then the results presented here add to the evidence that porin is not important in immunity against pertussis, because it can be assumed that the organism would only change antigens that had a strong immune response raised against them.

Unlike Omp40, CyaA is very large. It is a 1706 amino acid, 177-kDa protein that is divided into four domains (Figure 5.4); the catalytic domain (amino acids 1 to 400), the hydrophobic domain (amino acids 500-700), the glycine/aspartate-rich repeat domain (amino acids 1000-1600) and the C-terminal secretion signal domain (amino acids 1600 to 1706).

To focus our search, we wanted to screen just those areas of the sequence that might be immunodominant epitopes. A recent paper by Lee *et al.* mapped the immunodominant epitopes of CyaA, using monoclonal antibodies (MAbs). They found that 6 out of 12 MAbs tested reacted with the repeat region, suggesting that it may be immunodominant, and four MAbs bound to amino acids 373 to 828 (Lee, S.-J., 1999). MAbs that bound to amino acids 373 to 399 could inhibit accumulation of cAMP by more than 95% when they were incubated with CyaA which was then introduced into JurkaT-cells (Lee, S.-J., 1999). In addition, MAbs binding to amino acids 399 to 623 and 781 to 828 partially blocked cAMP accumulation, but also interfered with lysis of sheep erythrocytes. Based on these results, we decided to screen the DNA sequence encoding two regions: amino acids 373 to 828, which encompasses the tail end of the catalytic domain and all of the hydrophobic domain, and a second region, which encodes amino acids 888 to1706, containing the repeat region and the C-terminal secretion signal (Figure 5.4).

The same approach was taken for CyaA as was for Omp40. Primers were made in a step-wise fashion along the sequence with the reverse primer of the pair before overlapped with the forward primer of the pair that follows and so on (Figure 5.5). The primers used are shown in Table 5.2. The resulting PCR products were all between 250 and 400 bases in length. DNA from the seven *B. pertussis* strains were amplified with the fifteen CyaA primers using the PCR protocol for pertactin.

Region 1 (aa 373-828) was screened using primers AC4F, 4R to AC 7F, 7R. Figure 5.6 shows the SSCP patterns for primer pairs AC 4F 4R (A), AC 5F 5R (B), AC 1,2F 1,2R (C), and AC 3,4F 3,4R (D). No sequence variation was found in these regions.

The SSCP patterns for primers AC 5,6F 5,6R, and AC 7F 7R are shown in Figure 5.7, A and B respectively. There was no sequence variation found in this region either.

The next group of primer pairs, AC 8 to AC 16, includes the second region of interest, amino acids 888 to 1706. Figure 5.8 displays the SSCP patterns for AC 8F 8R (A), AC 9F 9R (B) and AC 10F 10R (C). The sequence flanked by primers AC 8F and 8R showed sequence variation in two of the seven strains tested, CCL-2 and CCL-4 (Figure 5.8A). Primers AC 8F 8R extend from amino acid 861 to 994 and include the beginning of region 2 that starts at amino acid 888, and the beginning of the repeat region at amino acid 913.

Figure 5.9 shows the SSCP patterns for AC 11F 11R (A), AC 12F 12R (B), and AC 13F 13BR (C), and Figure 5.10 shows the SSCP pattern for the remaining three primers, AC 14F 14R (A), AC 15F 15R (B), and AC 16F 16R (C). These primers encompass the remaining length of Region 2, amino acids 994 to 1706. No further sequence variation was found.

To investigate the nature of the mutation found in CCL-2 and CCL-4, using primers AC 8F and 8R, CCL-2 and CCL-4 were sent to be sequenced. They both contained the same mutation of a G to an A shift at position 3657 of the sequence, which resulted in a change from a valine to a methionine at residue 892. The significance of this mutation is unknown, but it is interesting that it was only found in the two strains, CCL-2 and CCL-4, which are potential vaccine strains. This would suggest that the adenylate cyclase found in the potential vaccine strains would be considered the "old" type while the adenylate cyclase found in the others strains would be the "new" type. It would be interesting to investigate whether strains circulating pre-vaccine and

immediately post-vaccine contain the same type of mutation in CyaA as the potential vaccine strains, and when the change from "old" to "new" began. These strains could probably be obtained from the Laboratory Center for Disease Control or possibly from Connaught Canada.

There were no major changes found in adenylate cyclase resulting in antigenic variants like the ones found in pertactin and the S1 subunit of pertussis toxin. The one modification that was found was a single base change that resulted in an amino acid change in two strains that are considered "old" and are potential vaccine strains. Therefore, this adenylate cyclase mutation could reasonably be considered "old" as well. This may be significant since it was found in an area that was bound by MAbs suggesting this area could be an immunodominant epitope (Lee, S.-J.,1999). The S1 pertussis toxin variants also differ by single base changes in the sequence resulting in amino acid changes supporting the notion that perhaps these are significant. If this change, from the adenylate cyclase mutation found in CCL-2 and CCL-4, to the type found in the other strains were significant, it may have resulted in a conformational change in the resulting protein so that it could not be recognized by immune defenses like antibodies. If the rate of change in adenylate cyclase is similar to the rate of change in pertactin and S1 pertussis toxin, and the "new" adenylate cyclase appeared the same time the "new" pertactin and S1 pertussis toxin variants did, then the two types of adenylate cyclase would be seen in the strains isolated between 1985-1994. Therefore, the adenylate cyclase change must have occurred earlier than pertactin and S1 pertussis toxin variants, possibly in the 1940s or 1950s. Further studies are the only way to determine whether

any of the mutations found in pertactin, the S1 subunit of pertussis toxin, and adenylate cyclase toxin are significant (see chapter VI).

Primers	Sequence (5′-3′)	Gene	Position
POR1F	GCATTGCTGCTCTTGTCACT	*Omp40*	406-426
POR1R	TCACCCAGATCTTCCGTACC	*Omp40*	692-672
POR2F	GGTACGGAAGATCTGGGTGA	*Omp40*	673-693
POR2R	TACGACGGGGTCTGGTACAT	*Omp40*	971-951
POR3F	GTCATGTACCAGACCCCGTC	*Omp40*	949-969
POR3R	AGAGCCAGCTTCACGACTTC	*Omp40*	1235-1215
POR4F	GAAGTCGTGAAGCTGGCTCT	*Omp40*	1216-1236
POR4R	GTAGGTGTAGCCCAGCGAGA	*Omp40*	1491-1471
POR5F	AGGCCAACTCGTACATGGTC	*Omp40*	1355-1375
POR5R	AATCGAAGCGGAACTTGC	*Omp40*	1636-1618

Table 5.1. Primers used to amplify the major porin gene (*Omp40*) and generate the SSCP patterns.

	Primers	Sequence (5′-3′)	Gene	Start Position (aa)
Region 1	AC4F	AGAACAATCCTTTCCCGGAG	*CyaA*	302
	AC4R	ACCCCATCAAGGCTGTCATA	*CyaA*	410
	AC5F	CTATGACAGCCTTGATGG	*CyaA*	403
	AC5R	GCCGAATTGCGTCATCAG	*CyaA*	486
	AC1,2F	GCCCTGATGACGCAATTC	*CyaA*	480
	AC1,2R	GACCGTTCCACCTGTCAACT	*CyaA*	580
	AC3,4F	GGATGTCGTTGACCGATGA	*CyaA*	552
	AC3,4R	GTCCAGCTGATCCGCATAGT	*CyaA*	637
	AC5,6F	AATCGCACTATGCGGATCA	*CyaA*	629
	AC5,6R	CCTGCAGGTTTTTCTCGAAG	*CyaA*	745
	AC7F	CGTACTTCGAGAAAAACCTG	*CyaA*	737
	AC7R	GATCTCGACGAATGTGGTG	*CyaA*	869
Region 2	AC8F	AGCG AATTCACCACATTCG	*CyaA*	861
	AC8R	ACATGGCGGTATTGGACATTC	*CyaA*	993
	AC9F	AATGTCCAATACCGCCATG	*CyaA*	987
	AC9R	GTGCACGTTGTACTTCAC	*CyaA*	1092
	AC10F	GAAGTACAACGTGCACCAG	*CyaA*	1087
	AC10R	GTCCTGCAGGAAGATGTCG	*CyaA*	1193
	AC11F	CACGCTGTATGGCGAGGAC	*CyaA*	1179
	AC11R	GATGAGCACATCCTTCATG	*CyaA*	1270
	AC12F	ACGTCATCGGTACGAGCATG	*CyaA*	1259
	AC12R	GTCTGGCTGTAATCGACG	*CyaA*	1357
	AC13F	CGTCGATTACAGCCAGAC	*CyaA*	1351
	AC13BR	TCCTGGAAGAACCAGTCGTC	*CyaA*	1467
	AC14F	GACTGGTTCTTCCAGGATGC	*CyaA*	1462
	AC14R	AGGCCATTGAGGACGTTGGC	*CyaA*	1558
	AC15F	CAACGTCCTCAATGGCCTG	*CyaA*	1542
	AC15R	TACCAGTCGTGCACGGTAAG	*CyaA*	1646
	AC16F	TACCGTGCACGACTGGTATC	*CyaA*	1640
	AC16R	AAGAACGCAAAGAGACGGCG	*CyaA*	1727

Table 5.2. Primers used to amplify Region 1 and Region 2 of the adenylate cyclase gene to look for sequence variations.

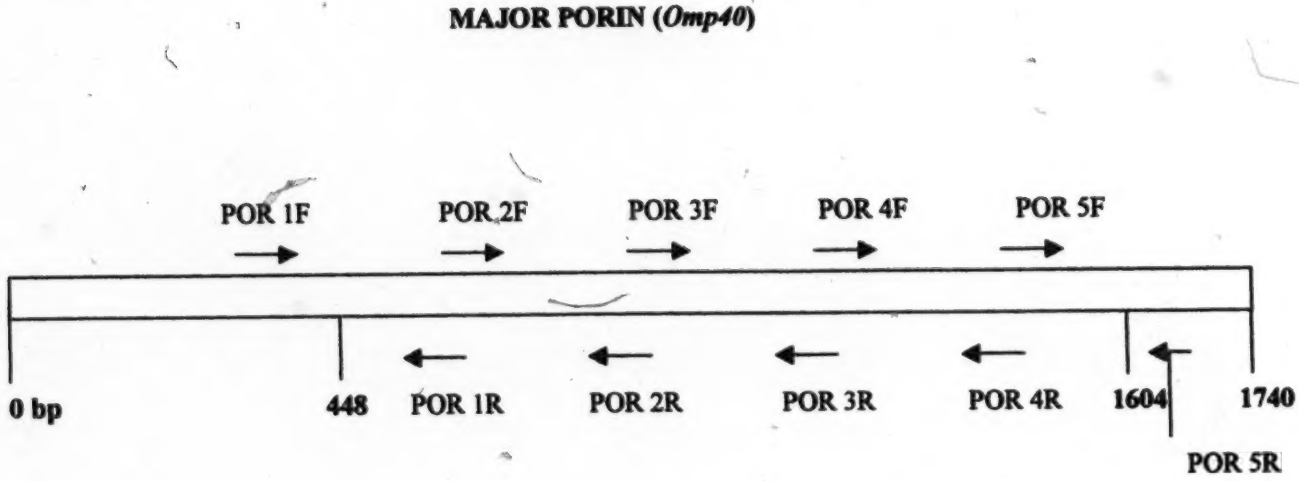


Figure 5.1. Schematic diagram of major porin. This diagram shows how the primers for SSCP screening of porin were chosen so the entire coding area was covered. The ORF for porin starts at base 448 and ends at 1604.

Figure 5.2. Shows the antigenic variation results for major porin. The gel in A shows the SSCP patterns resulting from primers POR 1F 1R, encompassing bases 406-692. The actual porin gene begins at base 448. There is no evidence of antigenic variation in this area. The gel in B shows the SSCP patterns for primers POR 2F 2R, containing bases 673-971. There is no evidence of antigenic variation in this area either. The gel in C shows the SSCP patterns resulting from primers POR 3F 3R, encompassing bases 949-1235. There is no evidence of antigenic variation in this area. The gel in D shows the SSCP patterns for primers POR 4F 4R, containing bases 1216-1491. There is no evidence of antigenic variation in this area either.

A.
CCL-4
CCL-3
CCL-2
CCL-1
BpeXba008
BpeXba003
BpeXba001
B.
CCL-4
CCL-3
CCL-2
CCL-1
BpeXba008
BpeXba003
BpeXba001
C.
CCL-4
CCL-3
CCL-2
CCL-1
BpeXba008
BpeXba003
BpeXba001
D.
CCL-4
CCL-3
CCL-2
CCL-1
BpeXba008
BpeXba003
BpeXba001

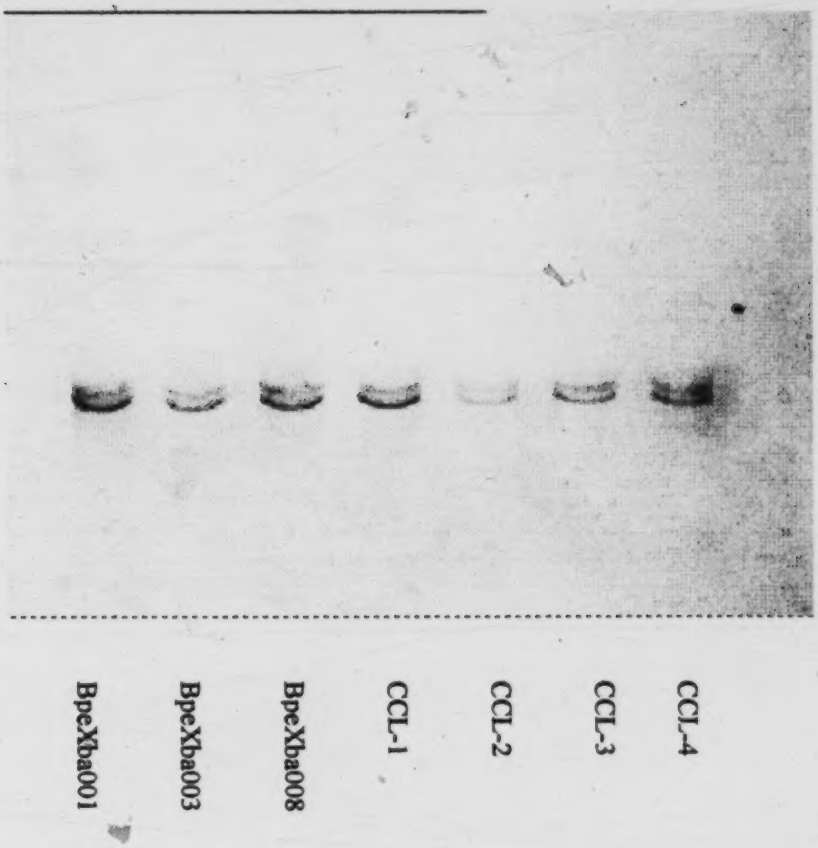


Figure 5.3. Shows the antigenic variation results for the last set of primers for major porin. POR 5F 5R includes bases 1355-1604 of the gene. There is no evidence of antigenic variation in this area.

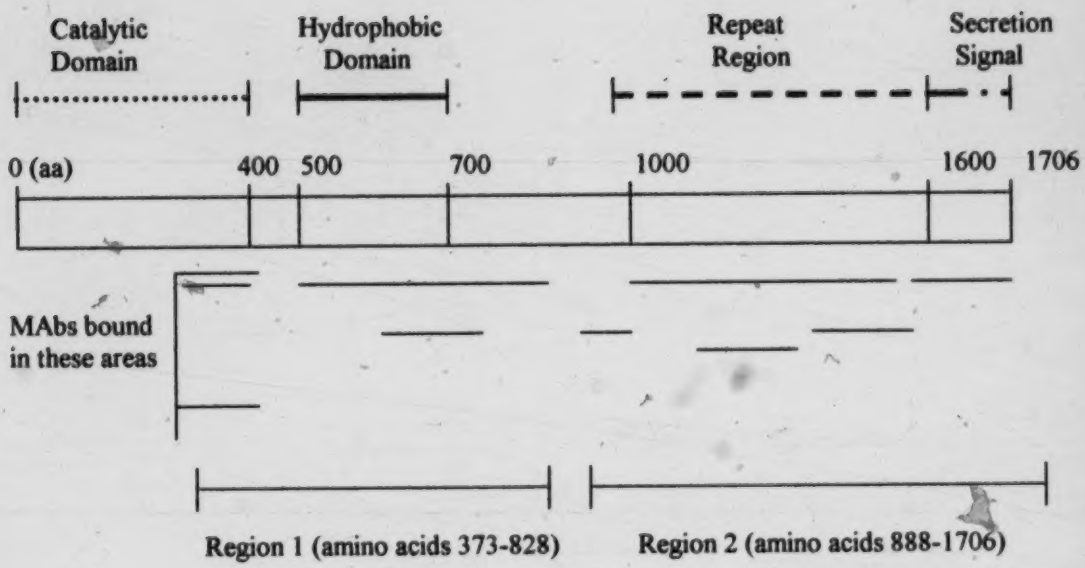


Figure 5.4. A diagram of the epitope map of adenylate cyclase generated by MAbs binding to the protein, taken from Lee *et al.*, 1999. The lines below the diagram show where the MAbs were found to bind. The areas we chose to screen, region 1 and region 2 were decided using this information.

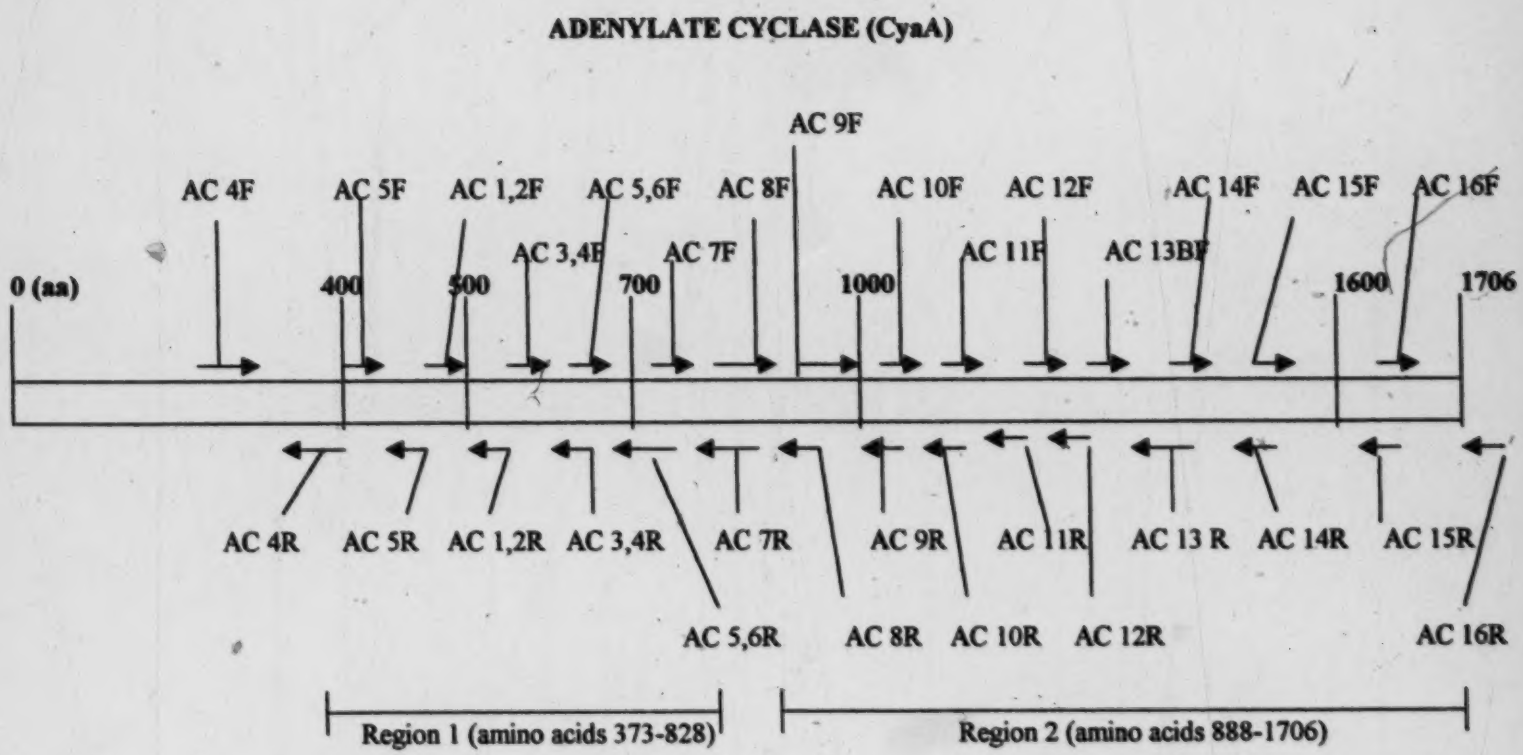
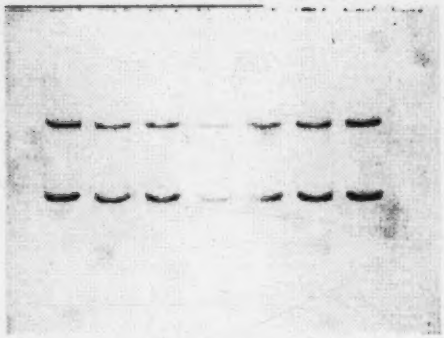


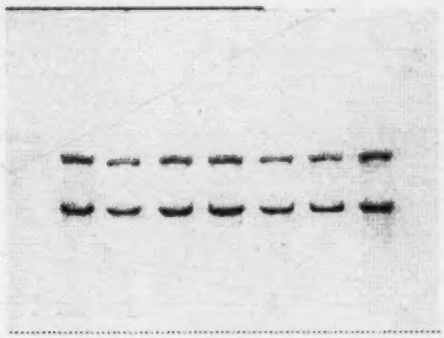
Figure 5.5. Schematic diagram of adenylate cyclase showing how the primers for SSCP analysis were chosen.

Figure 5.6. Adenylate cyclase antigenic variation in seven strains. Region 1 begins at residue 373 and ends at residue 828 (see Figure 5.4). Gel A shows the SSCP pattern for primers AC 4F 4R, ranging from residue 302-410, which include the start of region 1. Gel B shows the SSCP patterns for primers AC 5F 5R ranging from residue 403-486. Gel C shows the SSCP patterns for primers AC 1,2F 1,2R ranging from residue 480-580, and gel D shows the SSCP patterns for the seven strains from residue 552-637, primers AC 3,4F 3,4R. No sequence variation was present in this part of adenylate cyclase.

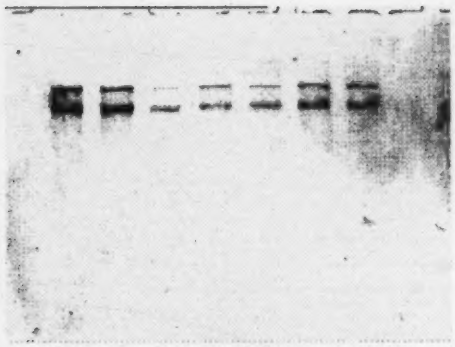
A.


BpeXbu001
BpeXbu003
BpeXbu008
CCL-1
CCL-2
CCL-3
CCL-4

B.


BpeXbu001
BpeXbu003
BpeXbu008
CCL-1
CCL-2
CCL-3
CCL-4

C.


BpeXbu001
BpeXbu003
BpeXbu008
CCL-1
CCL-2
CCL-3
CCL-4

D.

BpeXbu001
BpeXbu003
BpeXbu008
CCL-1
CCL-2
CCL-3
CCL-4

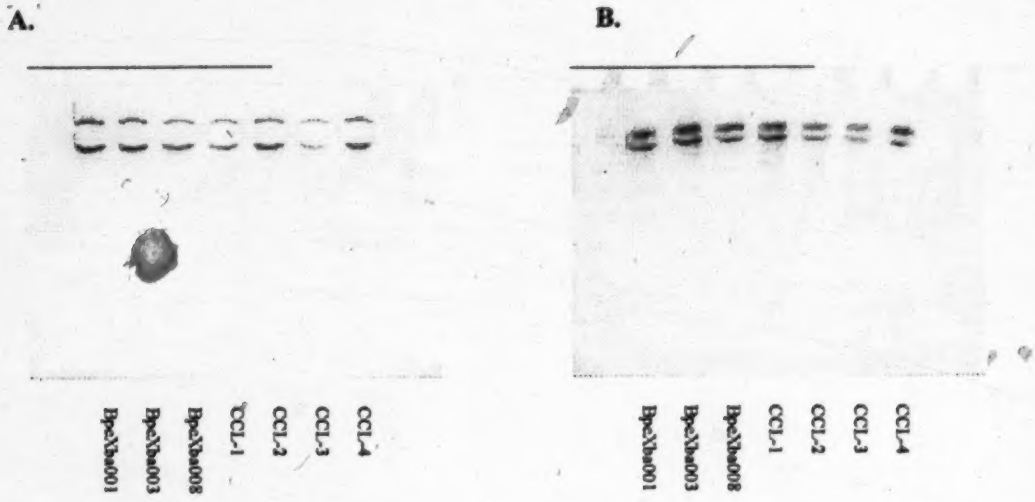


Figure 5.7. Adenylate cyclase antigenic variation in seven strains. Gel A shows the SSCP pattern for primers AC 5,6F 5,6R, ranging from residue 629-745. Gel B shows the SSCP patterns for primers AC 7F 7R ranging from residue 737-869. Region 1 ends at residue 828. No sequence variation was observed in the last part of Region 1.

Figure 5.8. Adenylate cyclase antigenic variation in seven strains. Region 2 begins at residue 888 and ends at residue 1706 (see Figure 5.4). Gel A shows the SSCP pattern for primers AC 8F 8R ranging from residue 861-993. Region 2 begins within this primer pair at residue 888, as does the Repeat Region at residue 913 (see Figure 5.4). Strains CCL-2 and CCL-4 have a different pattern than the other strains. Sequencing data showed a G to A base change resulting in a valine to methionine change at residue 892. Gel B shows the SSCP patterns for primers AC 9F 9R ranging from residue 987-1092, and gel C shows the SSCP patterns for primers AC 10F 10R ranging from residue 1087-1193. No sequence variation was found in the area covered by AC 9 to AC 10.

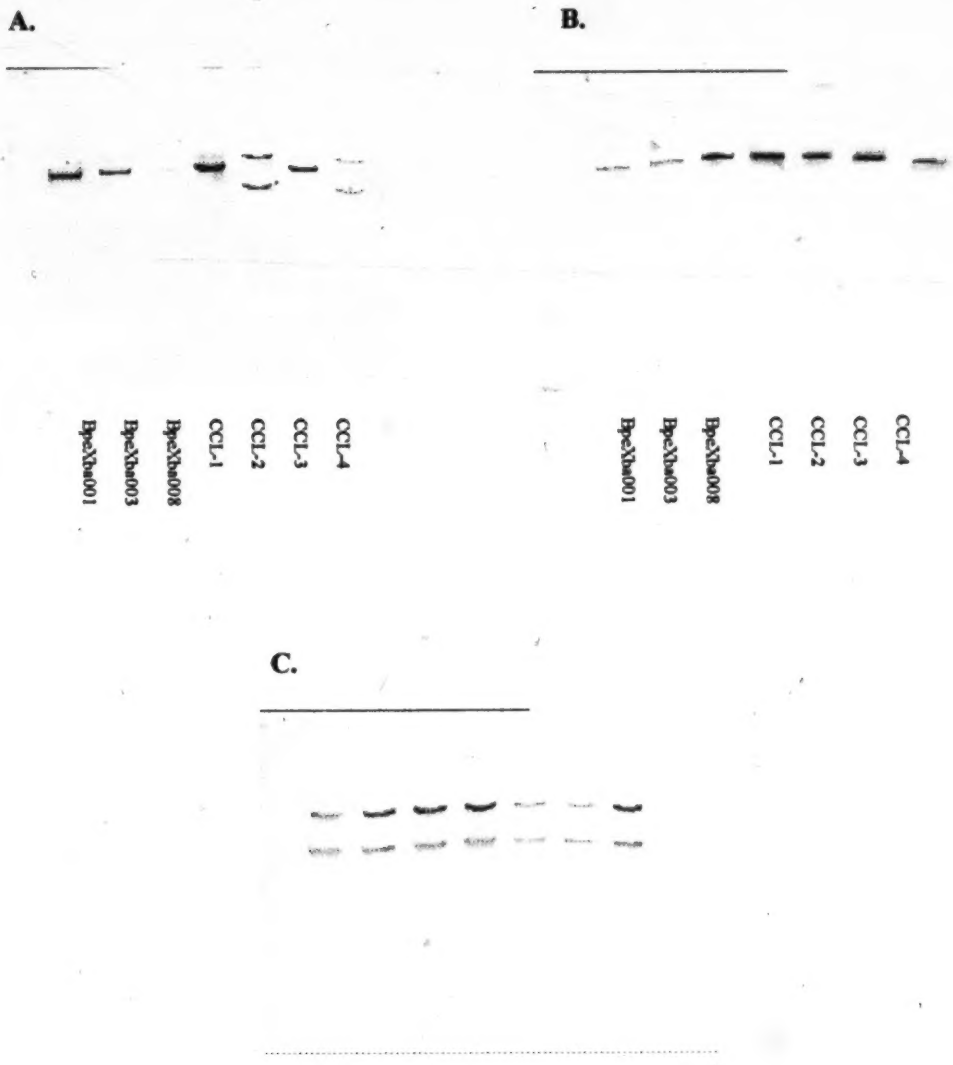

A.
BpeXba001
BpeXba003
BpeXba008
CCL-1
CCL-2
CCL-3
CCL-4
B.
BpeXba001
BpeXba003
BpeXba008
CCL-1
CCL-2
CCL-3
CCL-4
C.
BpeXba001
BpeXba003
BpeXba008
CCL-1
CCL-2
CCL-3
CCL-4

Figure 5.9. Adenylate cyclase antigenic variation in seven strains. Gel A shows the SSCP pattern for primers AC 11F 11R ranging from residue 1179-1270. Gel B shows the SSCP patterns for primers AC 12F 12R ranging from residue 1259-1357, and gel C shows the SSCP patterns for primers AC 13F 13BR ranging from residue 1351-1467. No sequence variation was found in this area of Region 2.

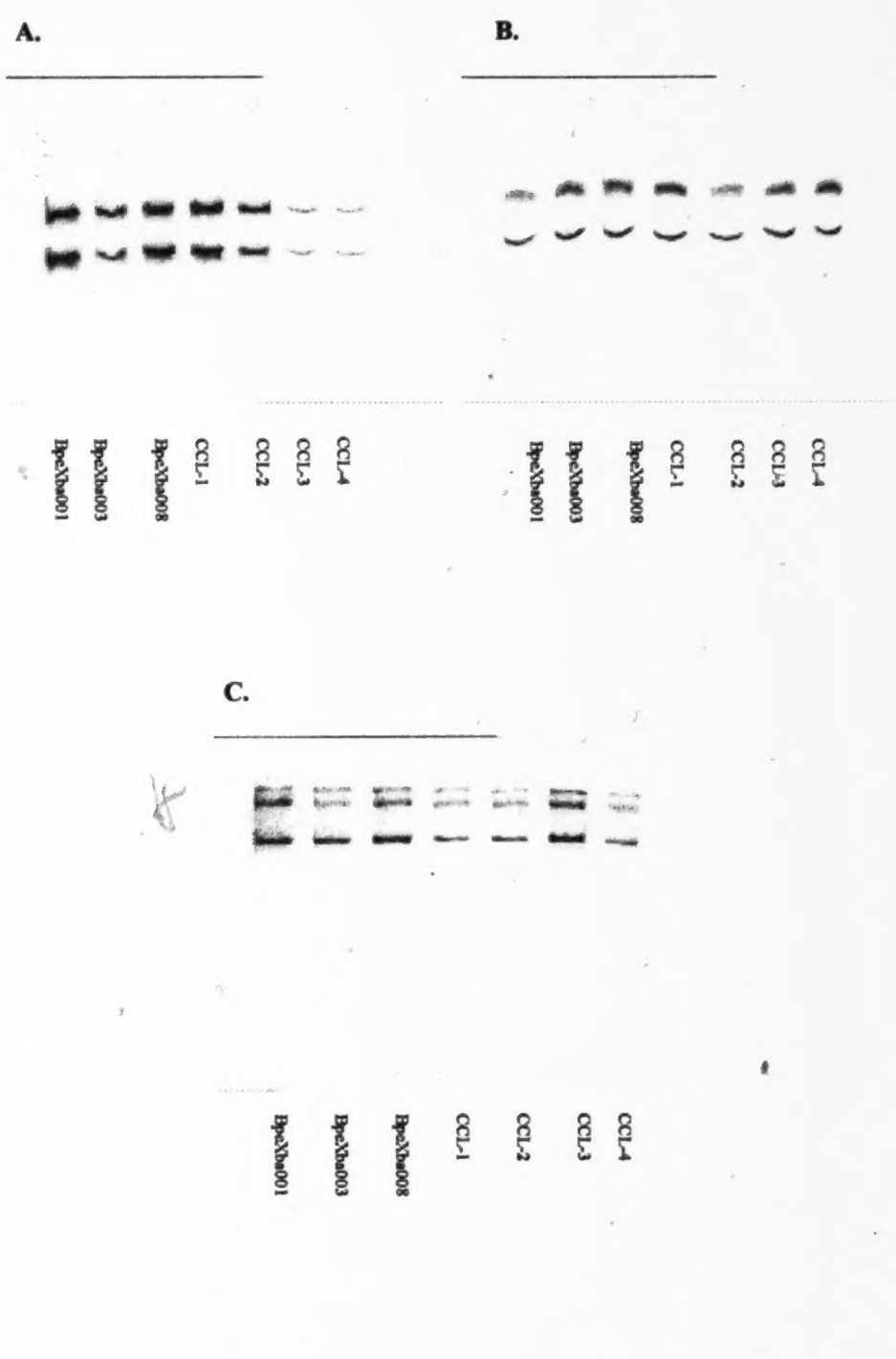
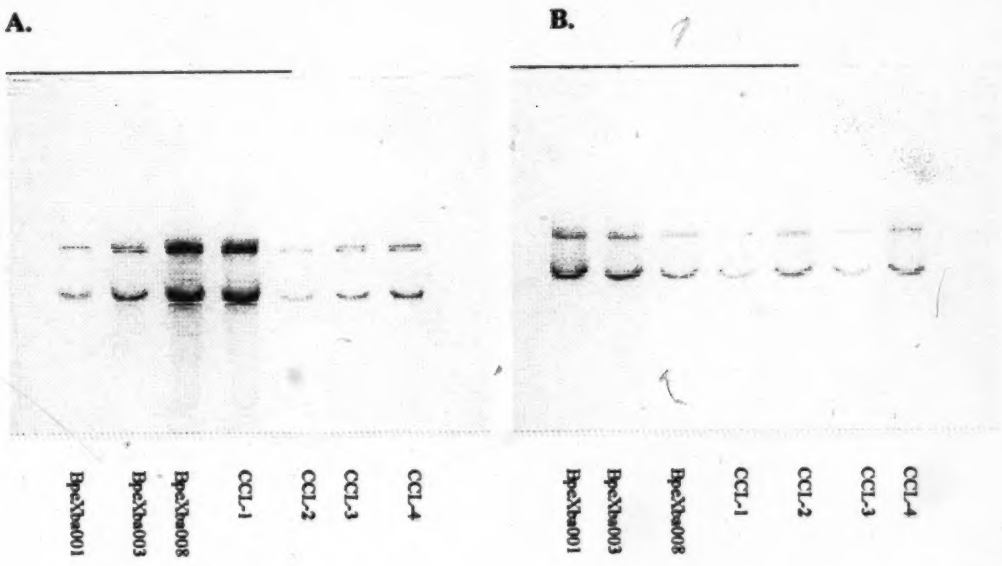
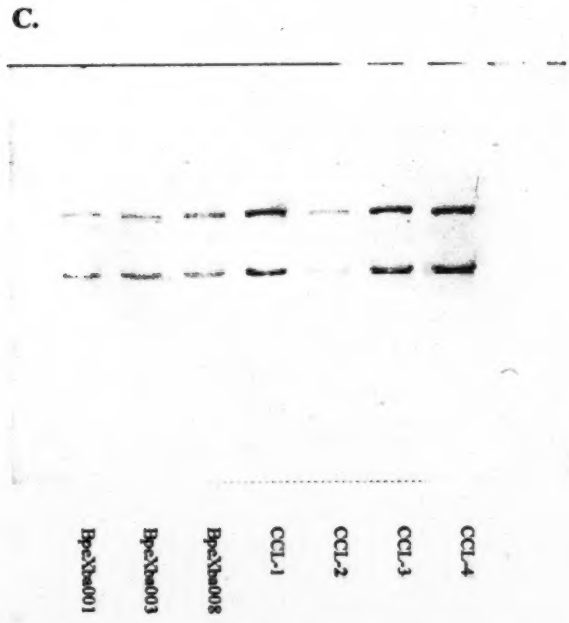

A.
B.
C.
CCL-4
CCL-3
CCL-2
CCL-1
BpeXba008
BpeXba003
BpeXba001

Figure 5.10. Adenylate cyclase antigenic variation in seven strains. Gel A shows the SSCP pattern for primers AC 14F 14R ranging from residue 1462-1558. Gel B shows the SSCP patterns for primers AC 15F 15R ranging from residue 1542-1646, and gel C shows the SSCP patterns for primers AC 16F 16R ranging from residue 1640-1727. No sequence variation was found in this last part of Region 2.


A.
BpeXbn001
BpeXbn003
BpeXbn008
CCL-1
CCL-2
CCL-3
CCL-4
B.
BpeXbn001
BpeXbn003
BpeXbn008
CCL-1
CCL-2
CCL-3
CCL-4


C.
BpeXbn001
BpeXbn003
BpeXbn008
CCL-1
CCL-2
CCL-3
CCL-4

CHAPTER VI

CONCLUSION AND FUTURE DIRECTIONS

The purpose of this thesis was to determine if there was vaccine-induced molecular evolution in *Bordetella pertussis* antigens, resulting in strains that are antigenically distinct from the vaccine strains. If this occurred, the immunity induced by the vaccine would potentially be ineffective against the antigenically distinct strains and might be part of the reason why there is an increase of pertussis in developed countries.

We looked at strains isolated in Alberta and Québec between 1985 and 1994. An epidemiological study was being conducted in our lab using pulsed-field electrophoresis (PFGE) as a tool to epidemiologically group these *B. pertussis* strains. There was a whooping cough outbreak in Alberta from 1989-1991 and its incidence began to rise in Québec in 1990. It was thought that PFGE coupled with antigenic- typing could shed some light on why the incidence of *B. pertussis* is increasing. In retrospect, a representative sample of strains previous to 1985 would have been helpful, but these were not available.

Initially, two antigens were looked at, pertactin and the S1 subunit of pertussis toxin, after evidence showing antigenic variants of these two antigens was found in The Netherlands (Mooi, F., 1998, Loo, 1999), Finland (Mooi, F., 1999), Italy (Mastrantonio, P., 1999), and the United States (Cassidy, P., 2000). We used single strand conformation polymorphism (SSCP) as a screening technique. To our knowledge, this is the first

application of SSCP to study the variation of antigenic alleles in a human pathogen. In our hands, it worked well.

Antigenic variants of pertactin and the S1 subunit of pertussis toxin were found in the strains circulating in Alberta and Québec between 1985 and 1994. There are currently 9 different pertactin types, that differ by the addition or deletion of the number of GGAVP and GGFGP repeats in Region 1, and by single base mutations in other parts of the gene. I found the ninth pertactin type here in Alberta and in Québec. The differences between the 8 pertactin types are shown in Figure 6.1. *Prn*9_1 differs from the rest of the pertactin types in that it contains 2 GGAVP repeats and 5 GGFGP repeats in Region 1. It is not known if it differs in any other areas of the gene. *Prn*9_1 appears sporadically in the ten-year period we studied. An analysis of the antigenic types of all strains or a representative sample from every pulsed-field type, from 1985-1994 would have to be done to determine when *prn*9_1 emerged.

Because of the unavailability of strains prior to 1985, we were unable to determine when the shift from "old" antigenic types to "new" antigenic types began. But, from the data presented in this thesis, it can be concluded that the period between 1985 and 1994 could be considered transitional. Strains containing "old" antigenic types, "transitional" antigenic types, and "new" antigenic types were all observed. When classifying the strains according to antigenic types, it might be more beneficial to group the two antigens, pertactin and S1 pertussis toxin together, and categorize the strains according to their antigenic profile, instead of looking at the antigens separately. It could be that the combination of the two "new" pertactin and S1 pertussis toxin antigens give the organism a better advantage rather than just with one "new" antigen. According to

Mooi *et al.*, by 1978, *ptxS1*A was already found in 80% of the isolates in The Netherlands (Mooi, F., 1998), therefore it must have appeared in the late sixties early seventies. The "new" pertactin types *prn*2 and *prn*3 did not appear in circulation until the early eighties. It was in the early eighties that pertussis really started to make its comeback in The Netherlands (Mooi, F., 1998). The same happened in the United States where *ptxS1*A appeared in 1970 and *prn*1 appeared in 1981 (Cassidy, P., 2000). From the data from The Netherlands and the United States, it is probable that the same thing occurred in Canada as well.

After 1967 the incidence of pertussis slowly started to increase in the United States and Canada (CDC Health Topics, Health Canada), consistent with the appearance of *ptxS1*A. In 1993, there was an 82% increase in the incidence of pertussis in the United States, compared to the year before and the highest incidence of pertussis in the United States since 1967 was in 1996 (Mahon, C., 2000). In Canada, the number of cases of pertussis increased to 8030 in 1990 from 2440 in 1989, resulting in an outbreak situation, and in 1995 there were 10 151 cases (Figure 6.2) (Health Canada). Interestingly, *prn*2/*ptxS1*A started to become more prevalent by 1993 in the U.S. and by 1996-1999 in the U.S., isolates with the "new" antigenic profile of *prn*2/*ptxS1*A comprising 98% of the strains tested (Figure 6.3) (Cassidy, P., 2000). In Alberta in 1990, the number of BpeXba001 isolates more than tripled from the year before, and the number of BpeXba002 isolates doubled (unpublished data). They both have the $prn2_1$/*ptxS1*A profile. Similar findings were present in Québec, although not to the same extent. The number of BpeXba001 isolates doubled from 1989 to 1990 while BpeXba002 remained unremarkable, and interestingly, BpeXba003 ($prn1_1$/*ptxS1*B) isolates started to increase

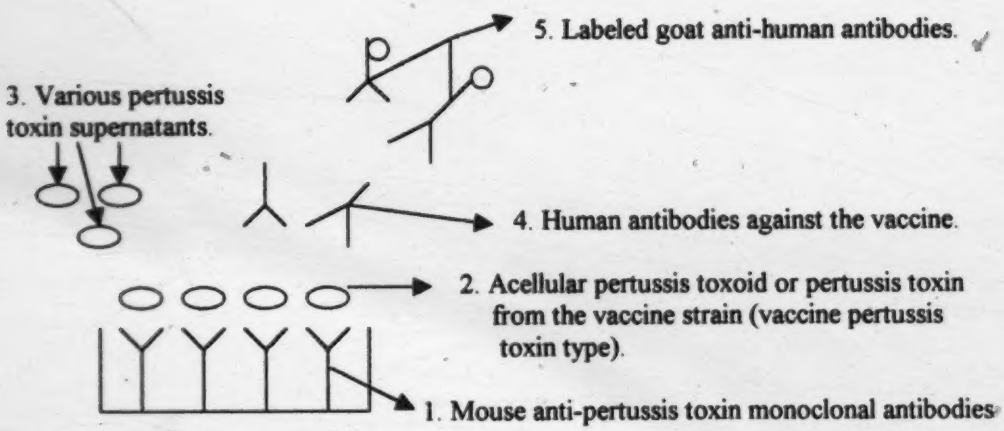
in 1988 with the highest number of isolates (80) in 1993 (data not shown). In Alberta, from 1995 to 1999, 84%, 86%, 89.6%, 91.7%, and 84.9% of strains isolated in the respective year contained the antigenic profile of *prn2/ptxS1*A (data not shown). It seems that the rise in pulsed-field types with the "new" profile in the early 1990s in Alberta and Québec coincided with the rise in pertussis. It could be that the combination of the "new" pertactin with the "new" pertussis toxin can evade vaccine-induced immunity better than just one new antigen can, and as the percentage of "new" isolates increase, so does pertussis.

Some conclusions can also be drawn regarding the antigenic composition of a single pulsed-field type, as presented in this thesis for BpeXba001, BpeXba002, BpeXba003, and BpeXba008 over the ten-year period. BpeXba001, BpeXba002, and BpeXba008 strains were all isolated in Alberta, while BpeXba003 strains chosen were isolated in Québec. It seemed that BpeXba001 and BpeXba002 followed a trend where strains isolated between 1985 and 1991 had a mix of "old", "transitional", and "new" antigenic profiles, and strains isolated after, from 1991 to 1994, had all "new" antigenic profiles (see Table 3.3 and Table 3.4, Chapter III), suggesting a selection of strains with the "new" profile. BpeXba008 did not appear until 1990; therefore all of the strains screened had "new" antigenic profiles (see Table 3.6, Chapter III). Together this BpeXba001, BpeXba002, and BpeXba008 data shows that pulsed-field types that have been circulating for at least 10 years will have mixed antigenic profiles, whereas the new pulsed-field types will be more consistent and have only one antigenic type. The inconsistency between pulsed-field type and antigenic type suggests the mechanism of genomic rearrangement and antigenic variation are independent of each other.

In the case of BpeXba003 the dominant pulsed-field type in Québec, all the strains tested were from Québec, and all had "old" antigenic profiles except for one. This could be the result of Québec using a different vaccine, the Armand-Frappier (A-F), than the rest of Canada until 1985. Based on the trends seen in The Netherlands and the United States, we could reason that the A-F vaccine had the antigenic profile *prn*1/*ptxS1*D. If this were the only profile in the vaccine, then there would have been pressure to switch to *ptxS1*B, but not to *ptxS1*A. Strains with *ptxS1*A were circulating but not to the same extent as in Alberta. Until Québec started using the same vaccine as the rest of Canada, would there be pressure to switch to *ptxS1*A. This would explain why a pulsed-field type with an "old" profile is the most common strain isolated.

A logical next set of experiments that need to be done is to determine if these pertactin and pertussis toxin variants have any biological effect. For example, can the immunity induced with vaccines made of "old" strains protect against the "new" or "transitional" strains? Is there a difference in how they protect against "old" strains? A study using the intranasal inoculation route to produce respiratory infections in mice has already shown that a tricomponent ACV was effective in clearing the lungs of all isolates regardless of their antigenic profile (Boursaux-Eude, C., 1999). But the aerosol infection model is superior to the intranasal inoculation model in its consistency, reproducibility, predictability, uniformity, and histopathology of infection (Halperin, S., 1988, Shahin, R. D., 1994). Keeping this in mind, the results of the intranasal study should be regarded with caution until further studies are done. The better experiment would be to have immunized mice infected by the aerosol route with the strains that have the different antigenic profiles.

To complement the animal studies, a competitive binding assay could also be done, which could show what role antibodies have in protective immunity induced by pertactin and the S1 subunit of pertussis toxin. We have come up with a competitive ELISA assay that could be used for pertussis toxin and pertactin that may eliminate the need to use phage display or synthetic peptides to raise antibodies against the different pertussis toxin and pertactin types.


5. Labeled goat anti-human antibodies.
3. Various pertussis toxin supernatants.
4. Human antibodies against the vaccine.
2. Acellular pertussis toxoid or pertussis toxin from the vaccine strain (vaccine pertussis toxin type).
1. Mouse anti-pertussis toxin monoclonal antibodies

In step 1, the well would be coated with the mouse anti-pertussis toxin monoclonal antibodies, available from the FDA or possibly Connaught. The addition of acellular pertussis toxoid (acellular vaccine) or pertussis toxin harvested from the vaccine strain would be step 2. Regardless of which antigen is used in step 2, either will bind to the monoclonal antibody. The amount of the pertussis toxoid would be known by its % composition in the vaccine. If pertussis toxin harvested from the vaccine strain were used, its activity would be determined by the CHO cell assay to establish the number of units of pertussis toxin per microlitre. The 3rd step would be to add the various types of

pertussis toxins in the form of culture supernatants normalized by the CHO cell assay. Step 4 would consist of adding the human antibodies. These human antibodies would be against the vaccine and would be acquired from volunteers in the department willing to be immunized with the new acellular pertussis vaccine. The different pertussis toxin types from the culture supernatants added in step 3 will compete for binding to the human antibodies, added in step four, with the pertussis toxin added in step two. The last step would be to add the labeled goat anti-human antibodies. The amount of the human antibody that binds to the vaccine pertussis toxin type can then be determined. If the human antibody recognizes only the vaccine pertussis toxin type, then the signal should be high. If the human antibody recognizes the other pertussis toxin types the signal will be decreased because the human antibody bound to the pertussis toxin from the various supernatants will be washed away. The same type of ELISA assay can be used to determine if the pertactin antibody raised against the vaccine can recognize the different pertactin types.

Of the two experiments proposed, the competitive binding assay may be the more relevant assay because it suggests using human antibodies raised against immunization, and *B. pertussis* does not cause whooping cough in mice, so its relevance to the human situation is uncertain. Although there is no direct serological correlation with immunity, it is thought that antibodies do have a role in protective immunity that involves preventing initial adherence to ciliated epithelial cells with an early rapid clearance of bacteria (Mills, K. H. G., 1998, Redhead, K., 1993). If this initial step in elimination of the bacteria was found to be disrupted, then possibly protective immunity would be disrupted.

I also screened adenylate cyclase toxin and major porin for evidence of antigenic change. The entire gene of porin was screened and no antigenic variants were found in the seven strains studied. In the case of adenylate cyclase, because the gene is so big, only Regions 1 and 2 were analyzed (see Figure 5.4 in Chapter V). These areas were chosen after epitope mapping by Lee *et al.* showed that MAbs bound most often to these regions. Antigenic variation was found in two strains, CCL-2 and CCL-4. This sequence variation resulted in an amino acid change where both strains contained the same mutation, a G to A base change resulting in a valine to methionine amino acid substitute at residue 892. Considering that these two strains are possible candidates for the Canadian vaccine strains, the mutation would be consistent with the "old type", a classification based on our pertactin and S1 pertussis toxin typing. The other strains I screened have the "new type" of pertactin and S1 pertussis toxin (except for pertussis toxin), so presumably the sequence coding for valine in these strains would be "new". We are unsure if the mutation is significant, that is, if the "new type" imparts any advantage with regards to evading vaccine-induced immunity. Even if it were significant, it would only be useful in evading WCV induced immunity since adenylate cyclase is not included in any ACV formulations. With the ACV now being used, there will no longer be any selective pressure against adenylate cyclase causing it to change, but neither is there any chance to protect against CyaA.

The objective of this thesis, to determine whether vaccine induced antigenic variation had occurred in *B. pertussis* strains in Alberta and Québec between 1985 and 1994, has been met. Hopefully the data in this thesis can help shed light on understanding why the incidence of whooping cough is increasing.

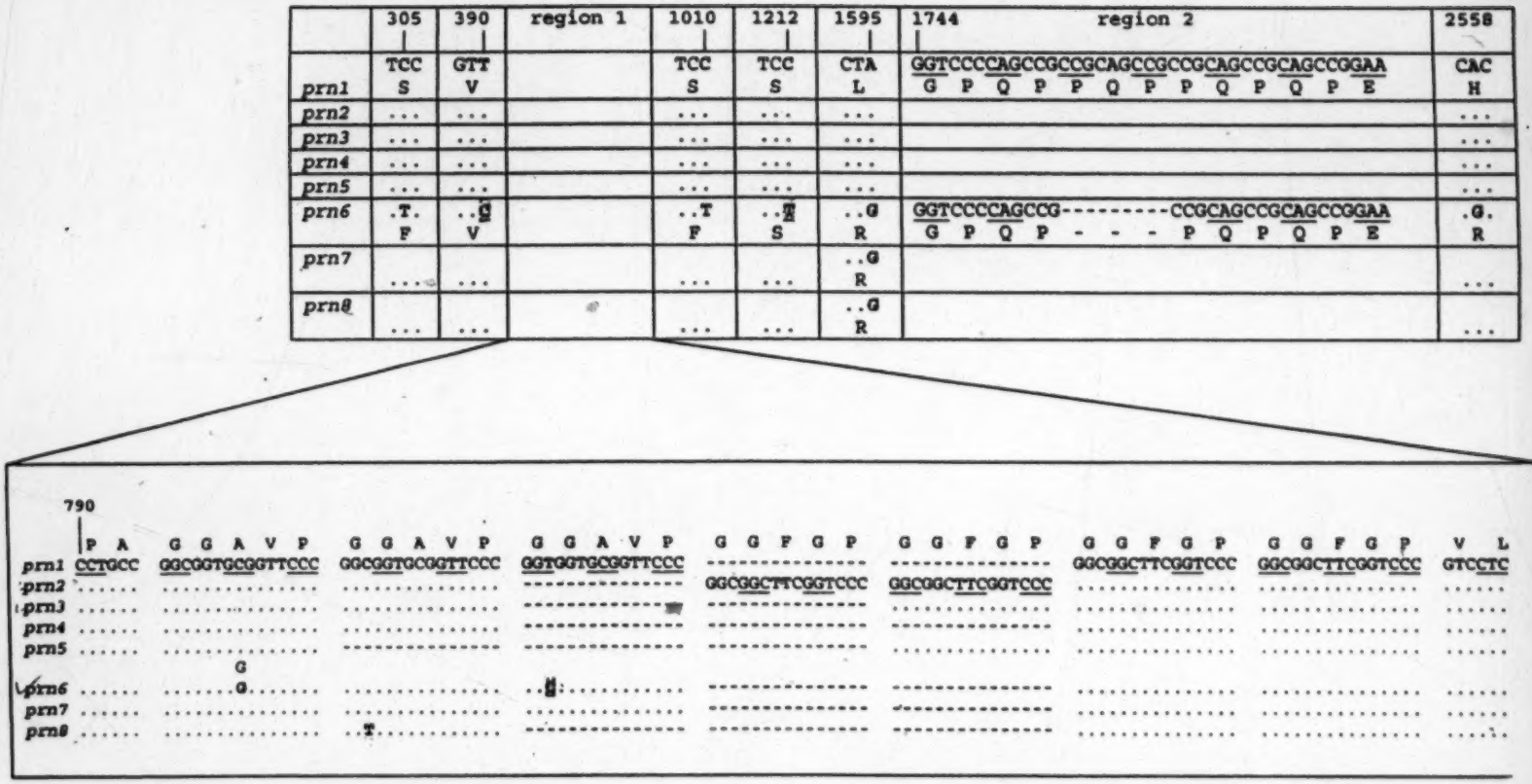


Figure 6.1. The polymorphic regions in the pertactin gene, taken from Mooi *et al.*, 2000. The different pertactin types differ in Region 1 by the number of GGAVP and GGFGP repeats and in other areas of the gene by single base substitutions. There were no differences in Region 2 except for *prn*6, from 18323, the mouse challenge strain. *Prn*9$_1$ (not shown) is so far known to differ in Region 1 only.

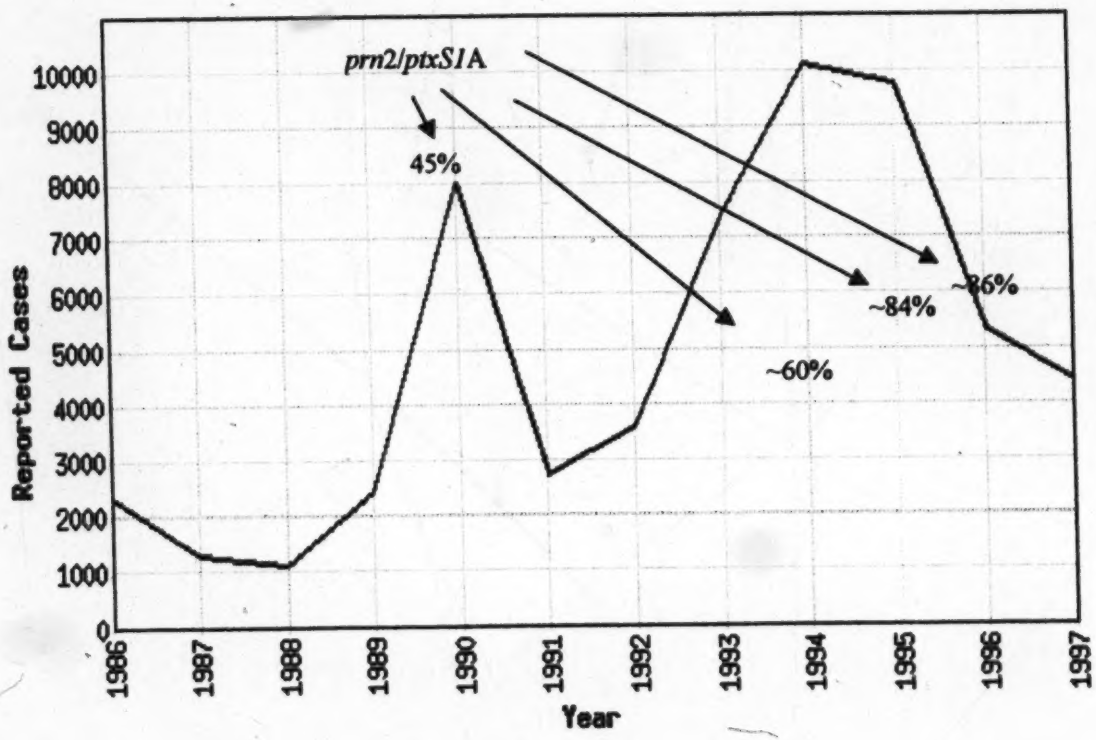


Figure 6.2. The incidence of pertussis in Canada from 1986-1997 (from www. http://www.hc-sc.gc.ca/hpb/lcdc/). This graph shows the approximate percentage of isolates that have the new antigenic profile of *prn2/ptxS1*A per year.

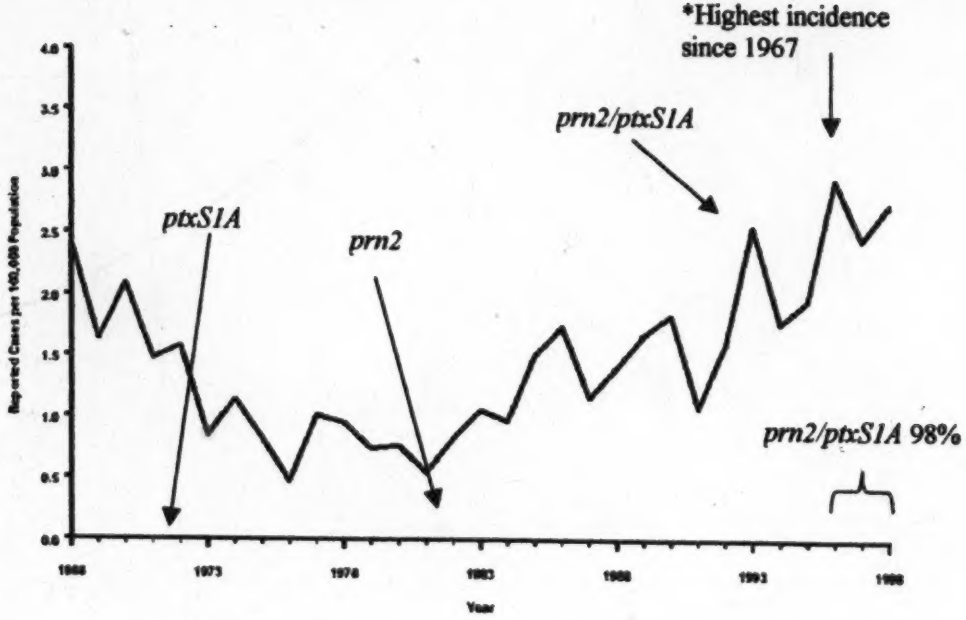


Figure 6.3. The incidence of pertussis in the United States from 1968-1998 (from www.cdc.gov). Pertussis toxin type A appeared in the early 1970s, while the new pertactin type 2 appeared in the early 1980s. In the United States, the new antigenic profile of *prn2/ptxS1A* did not appear until the 1990s.

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